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SOME SIMPLIFIED MATHEMATICAL TREATMENTS OF TRANSLOCATION IN PLANTS[1]

LEONARD HORWITZ[2]

DEPARTMENT OF BOTANY, STATE COLLEGE OF WASHINGTON, PULLMAN, WASHINGTON

When radioactive tracer techniques began to be used in studies on translocation in plants, experiments of a hitherto unparalleled degree of precision and incisiveness became possible. Many of the results obtained to date with isotopic techniques have merely been a confirmation, in a much more elegant way, of phenomena that had already been rather well established by other methods. But, data from tracer studies have lately been appearing in the literature which permit a much more quantitative evaluation of the translocation process than had previously been possible. The main feature of these data is that there is generally a more or less linear relationship between the logarithm of the radioactivity at any place in the stem and its distance from the region of application (1, 2, 3). In this paper the consequences of a number of simple theories of translocation have been derived mathematically, in order to see which of these theories are plausible and conform to experimental observations. In addition it is hoped that these analyses may make more clear the kinds of experiments necessary to define the character of the translocation process.

1. FLOW THROUGH A PIPE WITH IRREVERSIBLE LOSS THROUGH THE WALLS: At least two extant theories of translocation involve the movement "en masse" of all or a portion of the fluid contained in the sieve tube. These are the theories of protoplasmic streaming in which the movement is actuated by living protoplasm, and pressure flow in which osmotic pressure supplies the motive force (4). The latter theory will be dealt with in more detail in a later section. For the purposes of this model, translocation is visualized simply as involving unidirectional mass flow of the fluid contents of a pipe (the sieve tube column) regardless of the actuating mechanism. The presence of sieve plates will provide an additional obstacle to flow in the pipe, and may be of importance in deciding what actuating mechanism operates, but need not invalidate the use of a pipe as a model for the sieve tube column. Solute may diffuse through the walls of the pipe, but no net transfer of water occurs between the inside and outside of the pipe. Furthermore, although the flow is laminar, as is shown in appendix 1, there is assumed to be sufficient mixing so that concentrations within the pipe are essentially uniform throughout its cross section. In addition, attenuation of concentration at the front of flow of a solute, continuously injected into the pipe at some point, is assumed to be unimportant. Sufficient mixing might be obtained by diffusion and as a result of the interference to flow presented by the sieve plates.

In order to explain the experimentally observed distribution of radioactivity in short term translocation experiments, we consider the following situation. Suppose that for a distance h somewhere along the pipe, it is surrounded by a steady state concentration C_1 of the radioactive material X, and that K is a first order constant characterizing the rate of transfer of X through the pipe wall in the region h. If the velocity of flow of the fluid in the pipe is v and the pipe cross sectional area is A_p, then the concentration of X in the pipe at the outflow edge of the region h is

$$C_0 = C_1(1 - e^{-\frac{Kh}{A_p v}}). \qquad (1)$$

In order to account for X after it leaves the region h, we use the following additional symbols:

x = distance along the pipe from the region h in the direction of flow.

t = time.

C_p = concentration of X in the pipe at any distance x.

C_t = concentration of X in the tissue surrounding the pipe at any distance x.

k = constant for diffusion of X through the pipe wall or a first order chemical reaction rate constant for removal of X from the flowing stream into a bound form.

A_t = cross sectional area of tissue surrounding the pipe.

If there is neither diffusion of X out of the pipe nor net diffusion of water into the pipe, then there will be no attenuation of X concentration in the stem with distance away from the source region h. This obviously does not conform to experimental observations with P^{32}, C^{14}, and K^{42} (1, 2, 3).

However, if X may diffuse out of the pipe and is thereupon chemically bound into a species unable to diffuse back, then C_p will be subject to the following relation:

$$\frac{dC_p}{dx} = -\frac{k}{A_p v} C_p = -\frac{k_1}{v} C_p. \qquad (2)$$

[1] Received July 16, 1957.

[2] Present address: 1 Canyon Road, Berkeley 4, California.

Integrated, this gives:

$$C_p = C_0 e^{-\frac{k_1 x}{v}}. \quad (3)$$

The concentration, C_p, in the pipe, therefore, is not time dependent, and fulfills equation 3 from the end of region h to the front of flow of X. Beyond that it is zero.

Therefore, the concentration, C_t (which is in a bound form), in the tissue surrounding the pipe at a distance x, anywhere from region h to the front of flow of X, is given by:

$$C_t = \frac{k}{A_t} C_0 e^{-\frac{k_1 x}{v}} \left(t - \frac{x}{v}\right) = k_2 C_0 e^{-\frac{k_1 x}{v}} \left(t - \frac{x}{v}\right). \quad (4)$$

The total amount of X, then, at any level in the stem from region h to the front of flow is

$$X = A_p C_0 e^{-\frac{k_1 x}{v}} + A_t t k_2 C_0 e^{-\frac{k_1 x}{v}} - A_t \frac{x}{v} k_2 C_0 e^{-\frac{k_1 x}{v}}, \quad (5)$$

or

$$X = C_0 e^{-\frac{k_1 x}{v}} \left\{A_p + k\left(t - \frac{x}{v}\right)\right\}, \quad (6)$$

and

$$\log_e X = \log_e C_0 + \log_e \left\{A_p + k\left(t - \frac{x}{v}\right)\right\} - \frac{k_1 x}{v}. \quad (7)$$

The slope of the curve relating $\log_e X$ and x is

$$\frac{d \log_e X}{dx} = -\frac{k}{v\left(A_p + k\left(t - \frac{x}{v}\right)\right)} - \frac{k}{v A_p}, \quad (8)$$

and for a given value of x, decreases with time at the rate given by:

$$\frac{d^2 \log_e X}{dx\,dt} = \frac{k^2}{v\left(A_p + k\left(t - \frac{x}{v}\right)\right)^2}. \quad (9)$$

The rate of increase of $\log_e X$ with respect to time is

$$\frac{d \log_e X}{dt} = \frac{k}{A_p + k\left(t - \frac{x}{v}\right)}. \quad (10)$$

The rate of change of the slope with respect to distance and time in any region of the stem will decrease as this region falls farther behind the front of flow of X (that is, as $(t - x/v)$ increases).

That the formulations resulting from this analysis give a good representation of experimental data can be seen from figure 1, where equation 6 is plotted on semi-logarithmic coordinates. The following parameters, reasonable for the bean plants used by Biddulph and Cory (3), were assumed: $A_p = 0.1$ mm², and v = 500 mm/hr. With a value for k of 2 mm²/hr, curves result that are much like those obtained by Biddulph and Cory (3) with P^{32}. The steeper slopes for their curves of P^{32} distribution at short times are partially accounted for in equation 8 from which one would predict a somewhat greater slope at short times. However, one would also expect greater slopes at short times, due to the fact that X is only just beginning to diffuse into the region h around the pipe, and C_1 and therefore C_0 are still rising to their steady state values.

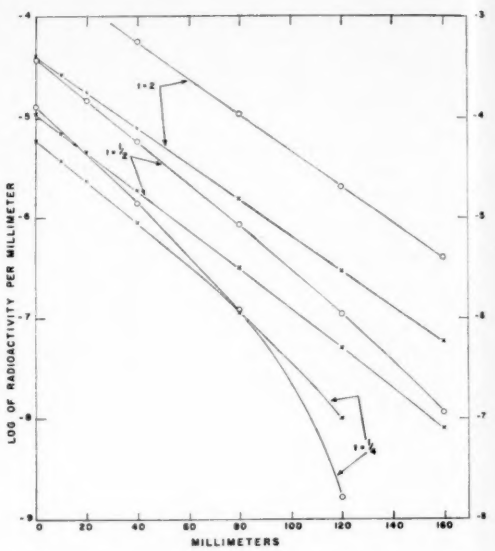

FIG. 1. Comparison of the radioactivity distributions obtained with models 1 and 3. Abscissa is distance along stem in millimeters. The ordinate is radioactivity per mm of stem in units of logarithm to the base 10. The curves through the crosses (×), referring to the right hand ordinate, are for model 1 with immediate attainment of the steady state in the source region h. They are, respectively, for ¼, ½, and 2 hours. The curves through the circles (○), referring to the left hand ordinate, are for model 3, with a half time of 24 minutes for attainment of the steady state in the source region h. They are also, respectively, for ¼, ½, and 2 hours. Other parameters, applicable to all curves, are $A_p = 0.1$ mm², k = 2 mm²/hr, and v = 500 mm/hr.

Swanson and Whitney (2) have made some interesting observations on the simultaneous translocation of two radioisotopes which can be interpreted in the light of the formulation developed here. In all cases, apparently, a plot of the logarithm of their radioactivities against distance from the point of application was linear. But whereas the slopes for Cs^{137} and P^{32}, applied simultaneously, were different, those for K^{42} and P^{32} were the same. Equation 8 shows that the

slope is determined by the three parameters k, A_p, and v. There is no need, therefore, to suppose, as Swanson and Whitney apparently did, that a difference in slope necessarily implies a difference in the rate of flow v, since differences in k or A_p can adequately account for it.

However, the data comparing K^{42} and P^{32} cannot be resolved, in the present formulation, without assuming completely different modes of transport for K^{42} and P^{32}, i.e., different A_p's or v's or both different. For although the slopes for these two substances remained the same, the rates of increase of the logarithms of their radioactivity with respect to time were different. Therefore, according to equation 10 at least one of the parameters, k, v, or A_p must differ. But since the slopes are the same, equation 8 demands that at least two of these parameters must differ. So either the v's or the A_p's or both involved in the transport of potassium and phosphorus must be different.

2. Flow Through a Pipe with Reversible Loss Through the Walls: This situation is like that of the preceding model except that X is not bound into an inert form upon leaving the flowing stream, but is available for reentry into it. Using the same symbols as before, this can be formulated mathematically in the following way: For the contents of the pipe contained within any element dx, conservation of matter demands that

$$\frac{\partial C_p}{\partial t} = - v\frac{\partial C_p}{\partial x} - \frac{k}{A_p}(C_p - C_t) = - v\frac{\partial C_p}{\partial x} - k_1(C_p - C_t). \quad (11)$$

The first term on the right side accounts for change in concentration due to flow of fluid in the pipe and the second term for transfer between the inside and outside of the pipe.

For the tissue surrounding the pipe and contained within the element dx

$$\frac{\partial C_t}{\partial t} = \frac{k}{A_t}(C_p - C_t) = k_2(C_p - C_t). \quad (12)$$

The integration of these equations is far more difficult than the ones for the model with irreversible loss. Fortunately, an essentially similar problem has already been solved for the case of heat transfer between a fluid flowing in a pipe and the walls of the pipe (5). Using these results and the following convenient arbitrary values for the parameters: $k_1 = 1\ hr^{-1}$, $k_2 = 1\ hr^{-1}$, and v = 200 mm/hr, the curves of figure 2 were drawn. They show the distribution of X in the stem after 6, 8, and 10 hours and are obviously far different from experimental curves.

The model with irreversible loss is essentially a special limiting case of the model with reversible loss, and the results of section I can also be obtained from equations 11 and 12. In the case of irreversible loss, they become

$$\frac{\partial C_p}{\partial t} + v\frac{\partial C_p}{\partial x} = - k_1 C_p \quad (13)$$

and

$$\frac{\partial C_t}{\partial t} = k_2 C_p. \quad (14)$$

By introducing two new variables

$$y = \frac{k_1 x}{v} \quad (15)$$

and

$$z = k_2\left(t - \frac{x}{v}\right), \quad (16)$$

they simplify to

$$\frac{\partial C_p}{\partial y} = - C_p \quad (17)$$

and

$$\frac{\partial C_t}{\partial z} = C_p. \quad (18)$$

Equation 17 integrates to

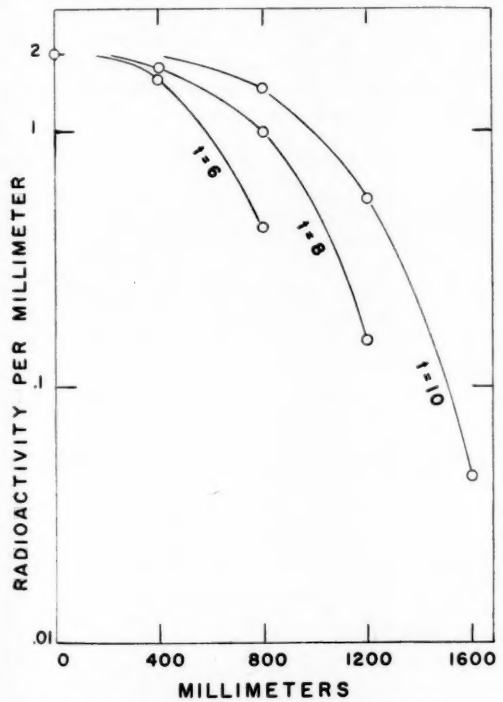

Fig. 2. Distribution of radioactivity with distance according to model 2. Abscissa is distance along stem in millimeters. Ordinate is radioactivity per millimeter along stem in arbitrary units on a logarithmic scale. The curves are for 6, 8, and 10 hours respectively. The other parameters, applicable to all curves, are $k_1 = 1\ hr^{-1}$, $k_2 = 1\ hr^{-1}$, and v = 200 mm/hr.

$$C_p = C_0 e^{-\frac{k_1 x}{v}}. \quad (19)$$

By substituting this value for C_p in 18, one obtains

$$C_t = k_2 C_0 e^{-\frac{k_1 x}{v}} \left(t - \frac{x}{v}\right). \quad (20)$$

Equations 19 and 20 are identical with 3 and 4 respectively.

3. Flow Through a Pipe with a Time Variable Source of Radioactivity: In the preceding models, the concentration, C_1, of the radioactive substance in the source region h surrounding the pipe, was assumed to be constant. Since this is obviously not always true and a non-steady state in the source region at short times was already invoked to explain certain features of the behavior of P^{32}, a quantitative treatment of a non-steady state situation is given here.

The model treated is one that might be useful in analyzing data, such as that of Vernon and Aronoff (1), who illuminated a leaf completely enclosed in an atmosphere containing $C^{14}O_2$ and followed the distribution of the radioactive translocate. The following symbols, in addition to those already defined, are used:

σ = rate of photochemical fixation of CO_2 in the source region h.
λ = isotopic ratio of $C^{41}O_2$.
K = constant for diffusion of C_1 from region h into the pipe.
A_h = average cross sectional area of the region h.
h = length of the pipe within the region h.

Distance is measured from the outflow end of the region h and is positive in the direction of flow.

If we assume that the flowing fluid in the pipe leaches X out of region h at such a slow relative rate that C_1 is essentially constant over the whole length h and that C_1 does not appreciably change in the time h/v, then the following equation holds in the length h:

$$\frac{dC_p}{dx} = \frac{K}{A_p v}(C_1 - C_p). \quad (21)$$

Integration of equation 21 gives:

$$C_p = C_1\left(1 - e^{-\frac{K}{A_p v}(x+h)}\right) = C_1\left(1 - e^{-\frac{K_1}{v}(x+h)}\right), \quad (22)$$

and at x = 0

$$C_0 = C_1\left(1 - e^{-\frac{K_1}{v}h}\right). \quad (23)$$

The rate of loss of X from region h into the pipe is from equation 23:

$$\nu = A_p v C_0 = A_p v C_1\left(1 - e^{-\frac{K_1}{v}h}\right). \quad (24)$$

Or, the rate of decrease in C_1 in the region h due to loss to the pipe is:

$$\nu_e = \frac{A_p v C_1}{A_h h}\left(1 - e^{-\frac{K_1}{v}h}\right). \quad (25)$$

(By expanding the exponential term in a Taylor's series around zero, it is easily seen that, as should be expected from the physical qualities of the system, $\nu_e \to C_1 K/A_h$ as $v \to \infty$.)

The rate of change of C_1, then, is given by

$$\frac{dC_1}{dt} = \sigma\lambda - \nu_e = \sigma\lambda - \frac{A_p v C_1}{A_h h}\left(1 - e^{-\frac{K_1}{v}h}\right). \quad (26)$$

Or, introducing the composite constants:

$$A = \frac{A_p}{A_h h} \quad (27)$$

and

$$\gamma = Av\left(1 - e^{-\frac{K_1}{v}h}\right), \quad (28)$$

we get

$$\frac{dC_1}{dt} = \sigma\lambda - \gamma C_1. \quad (29)$$

Integration of 29 gives

$$C_1 = \frac{\sigma\lambda}{\gamma}(1 - e^{-\gamma t}). \quad (30)$$

Upon substituting this value for C_1 in equation 23, we get an expression describing how the supply of radioactive material X, for translocation from the source region h, varies with time:

$$C_0 = \frac{\sigma\lambda}{Av}(1 - e^{-\gamma t}). \quad (31)$$

Now suppose, as in the first model, that X, in the course of flowing down the pipe, is lost irreversibly to the surrounding tissue. Then, the concentration, C_p, will be governed by equation 2, also as in the first model. In the present case, however, there is a time variable boundary condition, and integration of equation 2 gives

$$C_p = \left\{\frac{\sigma\lambda}{Av}\left(1 - e^{-\gamma\left(t - \frac{x}{v}\right)}\right)\right\} e^{-\frac{k_1 x}{v}}. \quad (32)$$

To calculate C_t as a function of x and t integrate

$$\frac{dC_t}{dt} = \frac{k}{A_t} C_p = k_2 C_p, \quad (33)$$

getting

$$\int_0^{C_t(t)} dC_t = k_2 e^{-\frac{k_1 x}{v}} \frac{\sigma\lambda}{Av} \int_{x/v}^{t} \left(1 - e^{-\gamma\left(\tau - \frac{x}{v}\right)}\right) d\tau \quad (34)$$

and

$$C_t = k_2 e^{-\frac{k_1 x}{v}} \frac{\sigma\lambda}{Av}\left\{\left(t - \frac{x}{v}\right) - \frac{1}{\gamma}\left(1 - e^{-\gamma\left(t - \frac{x}{v}\right)}\right)\right\}. \quad (35)$$

The content of radioactivity at any level in the stem, therefore, from region h to the front of flow is:

$$X = A_pC_p + A_tC_t, \tag{36}$$

or

$$X = \left\{k\left(t - \frac{x}{v}\right) + (1 - e^{-\gamma(t-\frac{x}{v})})\left(A_p - \frac{k}{\gamma}\right)\right\} \frac{\sigma\lambda}{Av} e^{-\frac{k_1 x}{v}}. \tag{37}$$

Equation 6 of the first model is clearly a limiting case of 37 when the arrival at the steady state is instantaneous, or when $\gamma = \infty$. When there is no loss from the pipe, i.e., when $k = 0$, then 37 reduces to:

$$X = \frac{A_h h\sigma\lambda}{v}(1 - e^{-\gamma(t-\frac{x}{v})}). \tag{38}$$

The slope obtained from plotting equation 37 on semi-logarithmic coordinates is

$$\frac{d \log_e X}{dx} = - \frac{\frac{k}{v} + \left(\frac{\gamma A_p}{v} - \frac{k}{v}\right) e^{-\gamma(t-\frac{x}{v})}}{k\left(t - \frac{x}{v}\right) + (1 - e^{-\gamma(t-\frac{x}{v})})\left(A_p - \frac{k}{\gamma}\right)} - \frac{k}{A_p v}. \tag{39}$$

The effect of the non-steady state situation on the family of curves obtained at various times can be succinctly demonstrated by a consideration of the slope at $x = 0$ and when the source region h has attained half its steady state concentration. The time to reach a half of the steady state concentration can be obtained from equation 30 and is $0.693/\gamma$. Upon substituting these values of t and x in equation 39, and simplifying, one obtains

$$\left(\frac{d \log_e X}{dx}\right)_{\substack{t=t_{1/2} \\ x=0}} = - \frac{\frac{k + \gamma A_p}{v}}{\frac{0.386k}{\gamma} + A_p} - \frac{k}{A_p v}. \tag{40}$$

Thus during the time when the source region is approaching its steady state, the slope will be greater by an amount that increases with γ, the time constant for approach. However, limits on the effect that γ can exert are imposed by itself, since a larger γ will not only increase the slope but will also decrease the duration of the increased slope by speeding up the approach to the steady state. As $(t - x/v)$ increases, i.e., as the region of the stem under consideration falls farther behind the front of flow, equations 37 and 39 approach the same values as 6 and 8.

These considerations probably preclude an explanation of the available data (1, 3), about attenuation of radioactivity with distance down the stem, that is based only on the non-steady state and does not make use of loss from the pipe as well. For, assuming a half time for attainment of the steady state of 24 minutes and a value of v equalling 500 mm/hr, one finds from equation 38 that, in the first 160 mm of translocation path, the attenuation of radioactivity is mostly well within one order of magnitude between 15 and 30 minutes and is rapidly decreasing. The experimentally observed degree of attenuation, however, is commonly around two to three orders of magnitude for P^{32} (3) and C^{14} (1).

A comparison of the curves obtained with respective half times, for attainment of the steady state, of 24 minutes ($\gamma = 1.73$) and 0 ($\gamma = \infty$) is shown in figure 1. The following additional parameters used are the same for the two curves: $A_p = 0.1$ mm^2, $v = 500$ mm/hr, $k = 2$. The non-steady state curve obtained at ¼ hour, where the front of flow is at 125 mm, is very similar to some of the C^{14} curves obtained by Vernon and Aronoff (1).

4. Osmotic Pressure Flow: The "pressure flow" theory of translocation (4) accounts for flow in the sieve tubes by the entrance of water into the translocation system under a high osmotic pressure at the end which supplies sugar, and its elimination under a low osmotic pressure at the end which receives sugar. The turgor pressure at the supplying end of the system must, therefore, be sufficient to, at the least, overcome viscous and other resistances through the length of the translocation column, to cause any required momentum increases of liquid in the pipe, and also to squeeze water out of the column against a small osmotic pressure at the receiving end. Although there are several detailed qualitative treatments of the pressure flow theory from a botanical point of view (4, 6, 7), there is no quantitative physical treatment. An attempt in this direction is made in what follows, with the hope that it may make clearer some of the factors involved in the pressure flow theory and their interaction.

A simple model, shown in figure 3, is considered. The left hand pipe, labelled S, contains an upper region in which sugar is produced by photosynthesis, an intermediate region analogous to the sieve tube column, and a lower consuming region in which sugar is removed by respiration or is transformed to an osmotically less active form. Adjacent to the pipe S and separated from it by a rigid membrane, permeable to water but not sugar, is the pipe Z (analogous to the xylem column), containing water, and connected to a water supply that is assumed to be limitless. The walls of both pipes are rigid.

Sugar produced in the photosynthetic region causes an osmotic influx of water which raises the pressure at the head of the column and causes a flow of sugar solution down the column, with water being squeezed below from pipe S into pipe Z. If the photosynthetic and sugar consuming rates are constant a steady state distribution of sugar concentration, pressure, and velocity of flow will eventually be achieved in pipe S. An approximate mathematical description of the steady

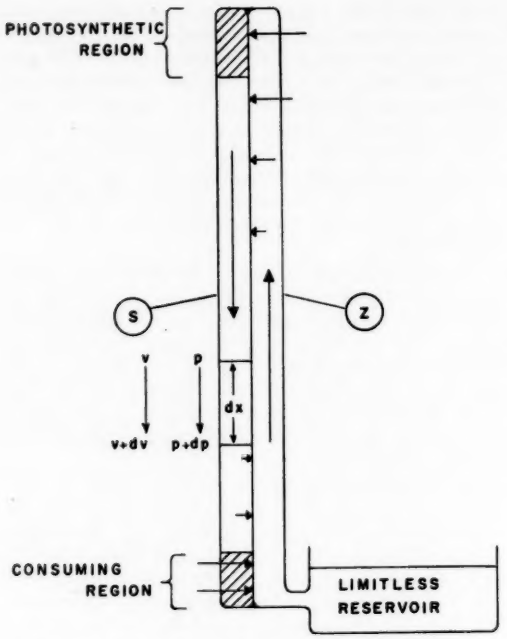

FIG. 3. Simple model for the osmotic pressure flow theory.

state system can perhaps be made in the following way.

The same symbols as were used previously are used here, and the distance x along the pipe is positive in the downward direction. The equation of continuity in pipe S, applicable outside the photosynthetic or consuming regions, states that the number of molecules of sugar passing any pipe cross section is constant, and is written

$$\frac{d(A_p v C_p)}{dx} = 0. \qquad (41)$$

If, as in several of the previous models, sugar is removed from solution in the pipe at a rate proportional to its concentration, then this equation is modified to

$$\frac{d(A_p v C_p)}{dx} = -kC_p. \qquad (42)$$

In the event that sugar is removed from the pipe at a rate independent of its concentration by an enzyme limited reaction (as, for instance, may occur when the phloem column passes alongside a uniformly active layer of cambium) then the continuity equation becomes

$$\frac{d(A_p v C_p)}{dx} = -\psi, \qquad (43)$$

where ψ is a constant.

Since, in the model, the walls of the pipe are rigid and the liquid is incompressible, any passage of water into or out of pipe S must be accompanied by a corresponding passage in the reverse direction somewhere. Therefore, the velocity of flow changes in response to entrance or exit of water. If we make the approximation that the density of sugar solution and water are equal, then the following expression is probably a reasonably good representation of the rate of entrance or exit of water:

$$A_p \frac{dv}{dx} = \alpha C_p - \beta(P_s - P_z), \qquad (44)$$

where α and β are constants and P_s is the pressure in pipe S and P_z is the pressure in pipe Z. This says that water enters the pipe at a rate proportional to sugar concentration in the pipe (in analogy to the approximate proportionality between sugar concentration and the equilibrium osmotic pressure) and leaves it at a rate proportional to the pressure difference between pipe S and pipe Z.

The pressure gradients in pipes S or Z can be given, at least approximately, by the following expression

$$\frac{dP}{dx} = -\epsilon v, \qquad (45)$$

where P is the pressure in the pipe and ϵ is a constant. The justification for the use of an expression of this type in the present system and the significance of ϵ are discussed in appendix 1.

By the use of any one of the three equations 41, 42, or 43, together with both 44 and 45 (applying to both P_s and P_z), one has the basis for a mathematical description of the system. However, these equations implicitly involve assumptions which are only approximations of greater or lesser reliability. For instance, it is taken for granted in equation 44 that, despite the occurrence of laminar flow, there is good mixing throughout the cross section of the pipe, and the possibility of variation in sieve tube properties along its length is completely ignored. Nevertheless, a further discussion of the model may make some of the features of osmotic pressure flow clearer.

Although it is designed to explain translocation in the phloem, the osmotic pressure flow theory involves a complete cycle of liquid transport down the phloem column and up again through the xylem. The force actuating this cycle is the turgor pressure of the chlorenchyma of the leaf, which, in the simple theory, must be sufficient to do the following: 1) push the flowing liquid through the plasmodesmata, sieve tubes, and sieve plates constituting the translocation column; 2) exert a turgor pressure on the inside of the membranes of the sugar consuming cells sufficiently greater than the equilibrium osmotic pressure of these cells so that water is forced out against the osmotic gradient at a rate necessary to account for the flow rate in the phloem; 3) push an amount of water up through the xylem equal to what has flowed down through the phloem. This last requirement cannot simply be accounted for as part of the ordinary rise of water in the transpiration stream for the osmotic pressure flow theory adds an extra transport burden to the xylem beyond that due to transpiration. One can explain

the extra flow in the xylem as due either to the turgor in the chlorenchyma forcing water through a complete cycle, or to an extra tension in the xylem water column. But these are equivalent since an extra tension in the xylem means that the turgor of the leaf chlorenchyma is correspondingly decreased.

In general one would expect from the simple pressure flow theory that the rate of flow varies with distance along the pipe, according to equation 44. The size of this effect, however, depends on the difference between terms involving concentration in the phloem and pressure. If the pressure dissipation down the pipe is paralleled by a corresponding decline in concentration (as by loss to adjacent tissue), there may be only a small variation in flow rate over long distances.

Crafts (10) has made the interesting suggestion that flow through the sieve plates, under the influence of osmotic pressure, does not occur through the microscopically visible sieve plate perforations, but rather through very small submicroscopic passages. He proposes that these passages have a diameter of about 150 to 450 Å. A quantitative evaluation of this proposal, however, indicates that it makes unacceptably large demands upon the osmotic pressure flow theory. Carman (11) has summarized the physical aspects of water flow through porous media which are used as the theoretical basis for the following calculations.

From measurements on Cucurbita (7), the following are used as reasonable dimensions: 0.05 cm for length of sieve tube element; 3×10^{-5} cm^2 for cross sectional area; 5×10^{-4} cm for depth of sieve plate. Assume that submicroscopic pores of diameter 4×10^{-6} cm occupy 50 % of the cross sectional area of the sieve plate, and an overall velocity of flow of 0.014 cm/sec (50 cm/hr) (3). The velocity of flow through the sieve plate, therefore, is 0.028 cm/sec. One can calculate the pressure drop through one sieve cell and one sieve plate, under these conditions, simply by applying the Hagen-Poiseuille equation (equation 46 in appendix 1). The flow through one sieve cell requires a pressure drop of only about 10^{-5} atmospheres. However, the pressure drop across one sieve plate, where the capillary cross section is about $4 \times 10^{-12} \pi$ cm^2, is one half an atmosphere. Even if the equation for laminar flow between parallel plates (8) is used, the pressure drop across a single sieve plate is still about one fifth or one sixth of an atmosphere. With two sieve plates every millimeter, it would be impossible to expect even an available turgor pressure of 20 atmospheres (10) to cause flow through the translocation column.

5. Cylcosis—Diffusion Transport and Activated Diffusion: Although simple diffusion cannot explain observed rates of translocation (4), it has been suggested (4) that diffusion across cell walls and sieve plates supplemented by rapid mixing within cells, due to cyclosis, might offer an adequate mechanism. Consider the sieve tube column as composed of sieve cells separated from one another by the sieve plates which are permeable to diffusion of the translocate, and suppose that cyclosis within the sieve cells is sufficiently rapid so that there is practically perfect mixing within each. An estimate of the extent of the increase in transport rate afforded by cyclosis can be obtained by deriving Fick's second law of diffusion (12) for these circumstances.

First, simply consider diffusion across one sieve plate. Distance across the sieve plate is measured along the x axis. According to Fick's first law of diffusion (12), the rate of transfer through a plane of unit area perpendicular to the x axis is approximately

$$F = -D^1 \frac{\Delta C_p}{\Delta x}, \tag{54}$$

where D^1 is the diffusion constant in the sieve plate, ΔC_p is the increase in concentration of translocate across one sieve plate, and Δx is the distance across one sieve plate. When there is a sieve cell adjacent to each sieve plate, equation 54 still expresses the rate of transfer across one sieve plate. However, this rate is also the rate of transfer across one sieve plate plus its adjacent sieve cell, since transfer of translocate across the sieve cell is assumed to be essentially instantaneous. The analogue of Fick's first law for transfer down the column composed of alternate sieve plates and sieve cells, then, is approximately

$$F = -(\omega + 1) D^1 \frac{\Delta C_p}{\Delta x}, \tag{55}$$

where ω is the ratio of the length of the sieve cell to the length of the sieve plate, and Δx is now the distance across one sieve plate plus one sieve cell.

Now the difference between the rate of transfer into and out of an element of the sieve tube column is proportional to the rate of change in concentration in the element. So

$$\Delta F = -\frac{1}{\omega + 1}\left(\frac{\omega}{\eta + 1} + 1\right) \Delta x \frac{\partial C_p}{\partial t}, \tag{56}$$

where η is the ratio of the volume of the sieve cell that does not contain translocate to the volume that does contain translocate. (Thus, if the sieve cell contained a large inert vacuole and cyclosis only occurred in a thin peripheral layer of cytoplasm, insulated by a membrane from the vacuole, then η would be the ratio of vacuolar to cytoplasmic volume.) Rearrangement of 56 gives

$$\frac{\partial C_p}{\partial t} = -\frac{\omega + 1}{\left(\frac{\omega}{\eta + 1} + 1\right)} \frac{\Delta F}{\Delta x}. \tag{57}$$

From equation 55 one gets

$$\Delta F = -(\omega + 1) D^1 \frac{\Delta^2 C_p}{\Delta x}, \tag{58}$$

which when substituted in 57 gives

$$\frac{\partial C_p}{\partial t} = \frac{(\omega + 1)^2}{\left(\frac{\omega}{\eta + 1} + 1\right)} D^1 \frac{\Delta^2 C_p}{(\Delta x)^2}. \tag{59}$$

And if we may pass to the limit, we obtain as the analogue of Fick's second law of diffusion in the sieve tube column,

$$\frac{\partial C_p}{\partial t} = \frac{(\omega + 1)^2}{\left(\frac{\omega}{\eta + 1} + 1\right)} D^1 \frac{\partial^2 C_p}{\partial x^2}. \qquad (60)$$

If $\eta = \omega - 1$ and if we assume that $\omega = 100$ (7), then the diffusion constant is effectively increased by a factor of about 5,000; if $\eta = 10\,\omega$, then the diffusion constant is effectively increased by about 9,000 times.[3]

The mechanism just discussed depends on ordinary diffusion processes subject to Fick's laws (12). However, if use is made of a system that preferentially allows diffusion in one direction, then even further increases in the rate of transfer can be imagined. This might be considered as an expression of the frequently mentioned theory of activated diffusion (13, 14). A theoretical model for such a system has been given (15), but, with it, analyses based simply on Fick's laws are probably no longer valid.

For the model where Fick's laws are applicable, one can make use of well known solutions of the diffusion equation. They will show how closely these models conform to actual data on the distribution of radioactive translocates. If a large amount of a radioactive translocate is applied to a region h surrounding the sieve tube column and it travels down the column by a diffusion mechanism without any of it leaving the column, then we want a solution of the equation

$$\frac{\partial C_p}{\partial t} = D \frac{\partial^2 C_p}{\partial x^2}, \qquad (61)$$

subject to the conditions

$$C_p = C_0 \qquad x = 0 \qquad t > 0 \qquad (62)$$

and

$$C_p = 0 \qquad x > 0 \qquad t = 0 \qquad (63)$$

The solution to this problem (12) is

$$\frac{C_p}{C_0} = 1 - \frac{2}{\sqrt{\pi}} \int_0^{\frac{x}{2\sqrt{Dt}}} e^{-\gamma^2}\, d\gamma$$

$$= 1 - \Phi\left(\frac{x}{2\sqrt{Dt}}\right) \qquad (64)$$

where $\Phi\left(\frac{x}{2\sqrt{Dt}}\right)$ is the error function of which extensive tables are available. The kind of distribution given by equation 64 is shown by the curve with the open circles in figure 4. It obviously does not conform to the known data (1, 2, 3).

Another possibility of interest is that, at the same time as the translocate is diffusing down the sieve tube column, it is also leaving the column at a rate

[3] An analysis of a similar type may prove of value in helping to understand the movement of growth substances through parenchyma tissue.

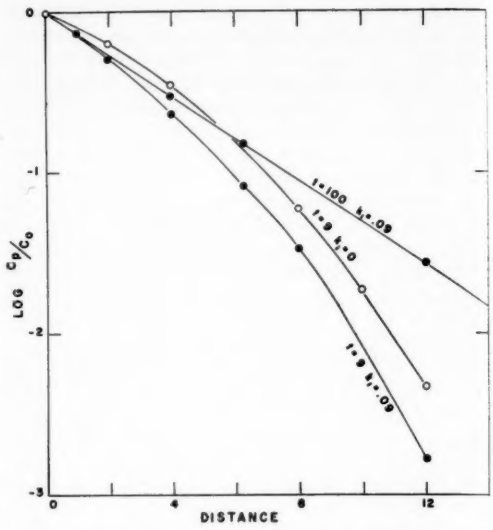

FIG. 4. Distribution of radioactivity with distance and time for two diffusion models. Abscissa is distance along stem in arbitrary units from a constant source of radioactivity. Ordinate is the ratio of concentration of radioactivity in the sieve tube column to that in the constant source in units of logarithm to the base 10. The curve through the open circles is for the model with no loss from the sieve tube column after 9 arbitrary time units. The curves through the closed circles are for the model with loss from the sieve tube column governed by $k_1 = 0.09$ and at 9 and 100 arbitrary time units. The diffusion constant, D, is unity in all cases.

proportional to its concentration in the column. The differential equation in this case becomes (12, p. 124)

$$\frac{\partial C_p}{\partial t} = D \frac{\partial^2 C_p}{\partial x^2} - k_1 C_p \qquad (65)$$

with the boundary conditions 62 and 63. Crank (12, p. 130) gives the solution of this problem, and the distributions obtained at two times are shown by the curves through the closed circles in figure 4. With increasing time the curves approach that at $t = 100$ and then do not change. These curves, of course, only account for the radioactivity in the phloem tube and not for that which has been lost irreversibly to the surrounding tissue. Assuming, however, that the shapes of these curves give an approximate representation of the shapes of the curves for total radioactivity (this assumption is certainly valid for longer times), then one can observe some striking similarities between the properties of this model and experimental data (1, 2, 3). At short times the rate of attenuation of radioactivity with distance is greater than it becomes later. At longer times, when C_p approaches its steady state value, the plots of the logarithms of total radioactivity versus distance are straight lines of constant slope and whose ordinate intercepts increase with time. This model, therefore, acts much like the

very first model considered in this paper, of irreversible loss from liquid flowing in a pipe, but at short times probably gives curves departing considerably from a linear relationship between the logarithm of total radioactivity and distance.

6. Surface Flow: A surface active substance accumulating at an interface causes a decrease in the surface tension. If the interface is mobile and only part of it is covered with surface active agent, then there will be a surface pressure difference between the covered and uncovered portion of the interface (equalling the surface tension difference between these parts) and surface flow will occur down the pressure gradient. Van den Honert (16) speculated about this phenomenon as a possible mechanism of translocation and performed some interesting experiments demonstrating transport at a liquid-liquid interface at rates that far exceeded diffusion rates. In order to approach a mathematical analysis of this mode of transport, one can make use of the observations of Crisp (17). He found that the velocity of transport is given by

$$\frac{dx}{dt} = -K_s \frac{\partial \Pi}{\partial x}, \tag{66}$$

where K_s is a constant depending on the properties of the surface active agent, the bulk phases, and the geometry of the system, Π is the surface pressure, and x is distance in the direction of flow. However, the rate of transfer through a line of unit length on the surface and perpendicular to the x axis is

$$F = C_s \frac{dx}{dt}, \tag{67}$$

where C_s is surface concentration of the transported material X. Then, from 66 we get

$$F = -K_s C_s \frac{\partial \Pi}{\partial x}. \tag{68}$$

If we suppose that the substance X behaves on the surface in a manner analogous to an ideal gas (as many surface active substances do under some circumstances (18)), then

$$\Pi = C_s k^1 T, \tag{69}$$

where k^1 is a constant and T is the absolute temperature. Also

$$\frac{\partial \Pi}{\partial x} = k^1 T \frac{\partial C_s}{\partial x}, \tag{70}$$

and upon substituting 70 in 68 we obtain an expression like Fick's first law of diffusion for the case where the diffusion constant is proportional to concentration:

$$F = -k^1 T K_s C_s \frac{\partial C_s}{\partial x}. \tag{71}$$

An analogue of Fick's second law (equation 61) is, then,

$$\frac{\partial C_s}{\partial t} = -\frac{\partial F}{\partial x} = k^1 T K_s \left\{ \left(\frac{\partial C_s}{\partial x} \right)^2 + C_s \frac{\partial^2 C_s}{\partial x^2} \right\}. \tag{72}$$

For the problem of transport in the phloem, this equation might apply under similar conditions as equation 61, where there is no loss from the phloem column to surrounding tissue. If substance X is lost to surrounding tissue irreversibly, however, then the following modification (analogous to equation 65) might apply:

$$\frac{\partial C_s}{\partial t} = k^1 T K_s \left\{ \left(\frac{\partial C_s}{\partial x} \right)^2 + C_s \frac{\partial^2 C_s}{\partial x^2} \right\} - k^{11} C_s. \tag{73}$$

The steady state solution of equation 65, describing diffusion down the sieve tube column at the same time as translocate is being lost irreversibly to the surrounding tissue, gives a result according well with experimental observations (2, 3). It is, therefore, of interest to see how well the steady state modification of 73, the analogous equation for surface flow, conforms to these data. An equation more amenable to analysis than 73 is obtained in appendix 2 and is

$$\frac{dC_s}{dx} = -\sqrt{\frac{2k^{11} C_s}{3k^1 T K_s} + \frac{c_1}{C_s^2}}, \tag{79}$$

where c_1 is a constant determined by the boundary conditions. However, the experimental data generally show a relationship between radioactivity and distance that can be described approximately by the relationship

$$\frac{dC}{dx} = -aC, \tag{80}$$

where C is radioactivity per unit length and a is a constant. This is quite different from 79, and, therefore, the simple model using surface flow does not appear to be a useful one for explaining translocation data.

Discussion

The characteristics of three main categories of mechanisms have been considered in this paper. They are 1), translocation by a continuous "en masse" flow through the length of the phloem column as is discussed in models 1, 2, 3, and 4; 2), cyclosis-diffusion transport discussed in model 5; and 3), surface flow as discussed in model 6. Under the assumption that translocate is absorbed irreversibly from the phloem column at a rate proportional to its concentration therein, one can nicely reproduce many important features of the experimental data by the use of flow type models and the cyclosis-diffusion model. The surface flow model is not so successful in this respect.

The valid use of a mathematical model is to help organize and interpret experimental data and to perhaps help direct the course of further investigation. Good quantitative data on translocation is relatively scarce and the models presented here are, therefore, not subject to comparison with a sufficiently broad range of experimental data to allow them to be accepted or rejected. Although the characteristics of much of the experimental data with radioisotopes can be reproduced by some of the models, there can arise

serious doubts about the "actuality" of the models studied. The mathematical analysis of models frequently involves gross simplification of the biological facts, and the types of models considered may be dictated largely by their simplicity and the mathematical skill of the author. In the present paper there are numerous simplifications and approximations, and a number of plausible models have not even been considered. For instance, the analysis of bidirectional flow within a single phloem strand was abandoned because of mathematical difficulties, and the cyclosis-diffusion model with a time variable source has not been presented.

It is possible to distinguish at least two markedly different possible mechanisms actuating "en masse" flow through the length of the phloem column (4). These are a) flow, such as that in osmotic pressure flow, and b) flow, like the flow in cytoplasmic streaming, that is actuated by the living protoplasm. Some possible modes of experimentally distinguishing the two may lie in these differences:

1) Pressure flow demands that there be a hydrostatic pressure gradient down the phloem column, while streaming flow does not require such a gradient.

2) In general, one would expect a flow velocity gradient along the phloem column in an osmotic pressure flow system, while streaming flow does not require this.

3) Osmotic pressure flow demands that there be a circulation of water in the xylem complementary to that in the phloem, while circulation by cytoplasmic streaming could occur completely in one phloem strand or from strand to strand. Clements (14) has criticized the osmotic pressure flow theory because of his failure to observe sufficient water movement in the xylem, though Crafts (9) believes these arguments have been adequately answered.

4) Streaming flow is dependent on a metabolic energy source, and, therefore, one might expect it to be more susceptible than pressure flow to interference with metabolism. However, pressure flow is dependent on the maintenance of a semi-permeable membrane, and this may also be dependent on normal metabolic activity. (In addition, it is possible that the pressure developed in a plant cell may be, at least in part, due to an active metabolic process (19, 20).)

5) Osmotic pressure flow precludes two way flow within a single phloem strand and perhaps also even within the stem taken as a whole. There is no such limitation with streaming flow.

6) Streaming flow demands that streaming occur in the phloem and apparently this has never been unequivocally observed (9, 21).

7) Streaming is known to be a phenomenon sensitive to even slight disturbances such as mechanical shocks, while pressure flow should be more resistant.

Summary

A number of models to explain the short term translocation patterns of isotopically labeled materials are analyzed mathematically:

1. "En masse" flow of translocate solution through a pipe with irreversible loss of translocate to the surrounding tissue.
2. Like model 1, except translocate is lost reversibly.
3. Like model 1, but the approach to the steady state in the region of isotope application is considered.
4. Osmotic pressure flow model.
5. Cyclosis-diffusion model.
6. Surface flow model.

Models 1 and 3 predict distributions of isotope that conform well with certain experimental results. Models 2 and 6 do not seem so successful in this respect. Model 4 is the most difficult to analyze, but the results indicate that, in general, one should expect both a velocity gradient and a hydrostatic pressure gradient along the translocation column. Analysis of model 5 indicates how much one may expect cyclosis to speed up the transport of solutes, and the predicted distributions of isotope from this model are close to certain experimental results; a possible contribution of an "activated diffusion" to this model is also mentioned.

Appendix 1, *Derivation of an Expression for the Pressure Gradient for the Pressure Flow Theory:* A number of writers (7, 9) on the pressure flow theory have made quantitative or qualitative use of the Hagen-Poiseuille equation to express the pressure gradient in the translocation pipe:

$$\frac{dP}{dx} = -\frac{8\pi\mu V}{A_p}, \qquad (46)$$

where μ is the viscosity of the flowing liquid. This equation is strictly applicable only in the case of laminar, non-accelerated flow in a circular tube of uniform cross section. Some justification, therefore, ought to be offered for its application to the sieve tube column where there is frequent interference to flow by the sieve plates and where, according to equation 44, there is acceleration of the flow due to entrance or exit of water through the pipe walls. In what follows the particular form of equation 46 is discarded, but it is concluded, nevertheless, that the pressure gradient can be reasonably accurately represented by an equation of the form

$$\frac{dP}{dx} = -\epsilon V, \qquad (45)$$

where ϵ is a constant.

In order to obtain an estimate of the magnitude of the Reynold's number for flow of a 10 % sucrose solution through the sieve tube column, use was made of sieve tube dimensions given by Crafts (7) for Cucurbita and translocation velocities given by Biddulph and Cory (3). Using 1.04 grams/cm^3 for density, 0.014 cm/sec (50 cm/hr) for velocity, 0.0050 cm for diameter, and 0.015 poise for viscosity gives a value of 4.8×10^{-3} for the Reynold's number in flow through the sieve tube lumen, and using 0.35 cm/sec for velocity and 0.00015 cm for diameter gives a Reynold's number of 3.6×10^{-3} for flow through the sieve plate

pores, assuming they are open and available for flow. These values are very far below the lower critical Reynold's number (8) and, therefore, flow in the sieve tube column is certainly laminar.

As a matter of fact these Reynold's numbers are so small that effects of viscosity should far outweigh accelerative effects. Experimental data given by Rouse (8, figures 85 and 129) indicate that at such low Reynold's numbers, the Euler number, determining the characteristics of flow under specific boundary conditions, is proportional to the square root of the Reynold's number. From the definition of the Euler and Reynold's numbers, therefore, one can deduce that even when the sieve tube pipe undergoes marked changes in form, as at the sieve plate, the dissipation of pressure is still proportional to the first power of the average flow velocity.

The contribution of the inertial effects, due to entrance or exit of water through the pipe walls, to the pressure gradient can, as a first approximation, be estimated by deriving a modified Bernoulli equation. First note, referring to figure 3, that gravitational force need not be a consideration since the flow is essentially no more than an eddy within a liquid, of uniform density, which is stationary as a whole. The distance, x, along the pipe S is positive downwards. The force acting on the element dx due to the pressure gradient is $-A_p dP$. From the considerations of the preceding paragraph, the viscous resistance is $-A_p \epsilon v dx$, where ϵ is a constant proportional to viscosity and v is the average rate of flow.

The momentum changes occurring in the element dx are due to acceleration from v to v + dv of liquid entering dx from the adjoining element above it, and also to acceleration from zero to v + dv of liquid entering dx by diffusion through the pipe walls. Since, for constant cross sectional area, the increase in velocity, dv, can be attributed entirely to influx of water through the walls, that fraction of the total liquid leaving dx which entered dx from the adjoining segment is $v/(v+dv)$. Therefore, the mass of water leaving dx in time dt which had to be accelerated from v to v + dv is

$$\frac{v}{v+dv}\rho A_p(v+dv)\,dt = v\rho A_p\,dt, \quad (47)$$

and the force necessary for the required acceleration is

$$v\rho A_p\,dt\,\frac{dv}{dt} = \rho A_p v\,dv, \quad (48)$$

where ρ is the density of the liquid.

The other part of the liquid leaving dx has a mass

$$\frac{dv}{v+dv}\rho A_p(v+dv)\,dt = dv\rho A_p\,dt \quad (49)$$

and the rate at which mass is added to the flowing liquid is, therefore,

$$\frac{dm}{dt} = \rho A_p\,dv. \quad (50)$$

The force necessary to cause the corresponding rate of change of momentum is

$$v\frac{dm}{dt} = \rho A_p v\,dv. \quad (51)$$

The relation between the four forces considered is the modified Bernoulli equation:

$$-A_p\,dP - A_p\epsilon v\,dx = 2\rho A_p v\,dv \quad (52)$$

or

$$\frac{dP}{dx} = -\epsilon v - 2\rho v\frac{dv}{dx}. \quad (53)$$

However, if one approximates the viscous resistance term on the right hand side by using the Hagen-Poiseuille equation and compares it to values of the second term calculated from known rates of translocation (3), then it is seen that only the viscous resistance term is important. Therefore, equation 45 should be a reasonable representation of the pressure gradient.

APPENDIX 2, *A Simplification of the Steady State Modification of Equation* 73: Equation 73, in the steady state, becomes

$$k^1TK_sC_s\frac{d^2C_s}{dx^2} + k^1TK_s\left(\frac{dC_s}{dx}\right)^2 - k^{11}C_s = 0 \quad (74$$

If we let $\eta = \frac{dC_s}{dx}$, then $\frac{d^2C_s}{dx^2} = \frac{d\eta}{dx} = \eta\frac{d\eta}{dC_s}$ and equation 74 can be put in the form

$$k^1TK_sC_s\eta\frac{d\eta}{dC_s} + k^1TK_s\eta^2 = k^{11}C_s, \quad (75)$$

or

$$k^1TK_sC_s\frac{d(\eta^2)}{dC_s} + 2k^1TK_s\eta^2 = 2k^{11}C_s. \quad (76)$$

Multiply through by $C_s dC_s/k^1TK_s$ and get

$$C_s^2 d(\eta^2) + 2C_s\eta^2\,dC_s = \frac{2k^{11}}{k^1TK_s}C_s^2\,dC_s \quad (77)$$

or

$$d(\eta^2C_s^2) = \frac{2k^{11}}{k^1TK_s}C_s^2\,dC_s. \quad (78)$$

Integration of 78 then gives as the acceptable result

$$\frac{dC_s}{dx} = -\sqrt{\frac{2k^{11}C_s}{3k^1TK_s} + \frac{c_1}{C_s^2}}, \quad (79)$$

where c_1 is the integration constant.

SYMBOLS USED

h: The distance along which the source region h, containing the reservoir of applied radioactive material, extends adjacent to the phloem tube.
C_1: The concentration of radioactive tracer in region h.
X: The name of the radioactive material.

K: The first order constant characterizing the rate of transfer of X from the region h into the phloem tube.
v: The velocity of flow of fluid in the phloem tube.
C_0: The concentration of X in the phloem tube at the outflowing edge of region h (at $x = 0$).
A_p: The cross sectional area of the phloem tube.
x: Distance along the pipe measured from the outflowing edge of region h and going in the positive direction away from region h.
t: Time.
C_p: Concentration of X in phloem tube at any distance x.
C_t: Concentration of X in the tissue surrounding the phloem tube at any distance x.
k: The first order constant characterizing the rate of loss of X from the phloem tube to the surrounding tissue.
A_t: The cross sectional area of the tissue surrounding the phloem tube.
k_1: k/A_p.
k_2: k/A_t.
e: Base of natural logarithm.
σ: Rate of photochemical fixation of CO_2 in the region h.
λ: Isotopic ratio of $C^{14}O_2$.
A_h: Average cross sectional area of the region h.
K_1: K/A_p.
ν: Rate of loss of X from region h into the phloem tube.
ν_c: ν/hA_h.
A: A_p/hA_h.
γ: $Av\left(1 - e^{-\frac{K_1 h}{v}}\right)$, is the time constant describing the approach to the steady state concentration of X in the region h.
ψ: A zero order rate constant for loss of X from the phloem pipe.
α: Proportionality constant between rate of osmotic flow of water and concentration difference across the semi-permeable membrane.
β: Proportionality constant between rate of flow of water through the semi-permeable membrane and hydrostatic pressure difference across the membrane.
P: Hydrostatic pressure in the xylem or phloem.
P_s: Hydrostatic pressure in pipe S (analogous to the phloem) in the osmotic pressure flow model.
P_z: Hydrostatic pressure in pipe Z (analogous to the xylem) in the osmotic pressure flow model.
ϵ: A constant, proportional to viscosity, that describes the relationship between the hydrostatic pressure gradient and velocity of flow.
π: Proportionality constant between circumference and diameter of a circle.
μ: Viscosity.
ρ: Density of liquid flowing in the xylem or phloem.
m: Mass.
D^1: Ordinary diffusion constant.
F: Diffusion or surface flow flux.
ω: Ratio of sieve cell length to sieve plate length.
η: Ratio of vacuolar volume to cytoplasmic volume in the sieve cell.
D: Diffusion constant in the cyclosis-diffusion model.
K_s: Proportionality constant between surface flow velocity and surface pressure gradient.
Π: Surface pressure.
C_s: Surface concentration at any distance x.
k^1: Proportionality constant relating surface pressure, concentration, and temperature.
T: Absolute temperature.
k^{11}: The first order constant characterizing the rate of loss of X from the phloem tube to the surrounding tissue in the surface flow model.

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EFFECTS OF INDOLEACETIC ACID ON THE UTILIZATION OF ACETATE-1-C^{14} BY PEA STEM SLICES[1]

JAMES F. NANCE
Department of Botany, University of Illinois, Urbana, Illinois

Studies on the utilization and evolution of acetaldehyde by plant tissues suggest that indoleacetic acid (IAA) may influence the partition of pyruvate and related products of glycolysis into various metabolic pathways (5, 6). Boroughs and Bonner (1) tested this possibility in their study of the effects of IAA on the metabolism of acetate-1-C^{14} by Avena coleoptiles. Except for a very appreciable stimulation of incorporation of activity into the non-cellulosic polysaccharides by IAA, they found no notable response to the auxin. It was demonstrated later that in wheat roots IAA significantly affects incorporation of activity from C^{14}-labeled pyruvate and acetate into organic acids, sugars, polyuronide hemicelluloses, pectic substances, lipides and cellulose (7, 8). Studies by Perlis (9), however, on the utilization of acetate-1-C^{14} by pea stem slices revealed experimental effects of doubtful significance.

Recent work in this laboratory indicates that effects of IAA on the metabolism of acetate by pea stem tissue can be demonstrated conclusively in experiments involving pre-treatment of the slices with IAA followed by brief exposure to the labeled substrate (4). Experiments of this sort are described below.

Materials and Methods

Pea seedlings (var. Alaska) were grown in the dark at 20° C on sphagnum saturated with 0.025 M $CaCl_2$ and were used for the experiments 8 days after the seeds were planted. Preliminary experiments had shown that stem sections from peas grown in this manner exhibit a more impressive growth response to IAA than those from seedlings given a more complex nutrient solution. Transverse slices, 2 mm in length, were cut from a section of the stem extending from about 2 to 12 mm below the epicotyl arch. The slices were washed, then centrifuged in a perforated container at a low speed to remove adhering water.

Acetate-1-C^{14} having a specific activity of 6.7 millicurie/millimole was the source of carbon 14. This material was available as the potassium salt and was used in this form for most of the experiments. For use in two experiments the salt was converted to the calcium salt by passing a solution of it through a column containing the calcium form of Duolite C-3. Most of the experiments of this study involve a pre-treatment period during which the stem tissues were incubated in various non-radioactive solutions. For pre-treatment the solutions and tissues were placed in Erlenmeyer flasks of one liter capacity and these were placed on a shaker to effect aeration of the solution. During the period of exposure to radioactive acetate the slices and solutions were in closed containers (Pyrex gas washing bottles, no. 31760). A short piece of capillary tubing replaced the fritted glass aerator usually found in this assembly. The outlet tube was attached to the inlet tube of a second gas washing bottle. This bottle, containing 0.1 *N* NaOH to trap CO_2, was equipped with a fritted glass aerator. Carbon dioxide-free air was supplied for the aeration of the slices. Fresh weights of tissues used in the experiments ranged from 3.25 to 7.0 g.

After exposure to radioactive acetate the washed slices were extracted with boiling, 95 % ethanol and then with an equal volume of boiling, 70 % ethanol. Three extractions with each solvent mixture were made and a total volume of 250 ml of extractant was used for each sample. This is the 1st step of a fractionation procedure which is outlined in figures 1 and 2.

Fractionation of the ethanol extract (fig 1) involved removal of the ethanol, after the solution was adjusted to pH 9.0 with NaOH, by vacuum distillation, acidification and extraction of the aqueous residue with ether for 72 hours by a method essentially that described by Isaacs and Broyer (2) and separation of the lipides from the organic acids in the ether extract by addition of water and titration of the acids with NaOH to pH 8.5. The ethereal solutions were dried down on a warm water bath and the residue taken up in chloroform. Activity in the chloroform solution was determined by the technique of direct plating, as were all other determinations of activity reported here. Details of the counting procedure are given below. The aqueous solution from the ether extract was brought to dryness on a hot water

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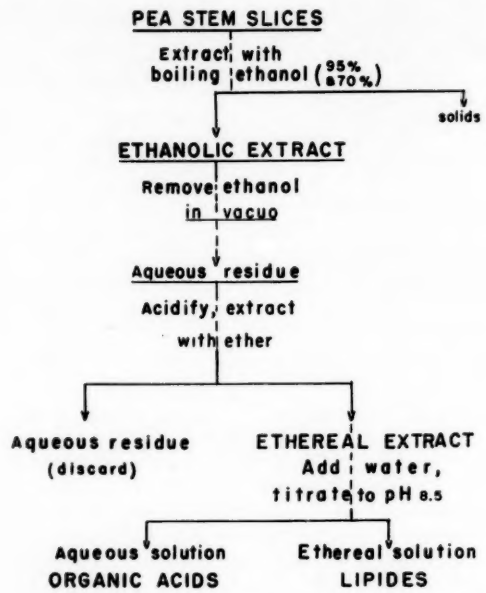

FIG. 1. Procedure for fractionation of pea stem slices. Separation of materials in the ethanolic extract.

bath. This residue was redissolved in water and aliquots assayed for activity.

The ethanol-extracted slices were placed in a Servall tissue homogenizer, covered with cold water and homogenized at 14,000 rpm for 5 minutes (fig 2). During the period of homogenization, the stainless steel vessel containing the tissue was immersed in an ice bath. Particulate and fibrous material of the homogenate was separated from a very cloudy, colloidal preparation by means of glass wool. Solids retained on the glass wool were resuspended in water and the mixture filtered once more through glass wool. These two aqueous preparations were combined, labeled "water extract," and assayed for radioactivity. This extract includes materials hydrolyzed by Takadiastase and by a hemicellulase of fungal origin. There is no solubilization of activity when the mixture is incubated with Pectinol R–10, a commercial pectinase preparation, suggesting the absence of pectic substances.

Isolation of the pectic substances of the cell wall materials was effected by repeated extraction of the solids held by the glass wool with 0.05 *N* HCl, followed by the precipitation of the pectic substances from the acid extract with ethanol, a procedure recommended by Kertesz (3). Each sample was extracted four times with 15 ml of 0.05 *N* HCl held at 80° C for 30 minutes. After each extraction the mixture was filtered through glass wool. To the pooled extracts enough 10 % HCl was added to make the concentration of the acid equal to 0.05 %. Precipitation of the pectic substances was accomplished by the slow addition of two volumes of 95 % ethanol. Separation of the pectic substances from the alcohol was made by centrifugation. The centrifugate was resuspended in an ethanol : water mixture (2 : 1, v : v) and recentrifuged three times to remove free acid. Finally, the washed pellet was suspended in water and aliquots of this preparation were assayed for activity.

Solids remaining after the extraction of the pectic substances were washed with water, dried and covered with 5 ml of 4 % NaOH and allowed to stand for 48 hours in a sealed desiccator, otherwise empty. The alkaline solution containing polyuronide hemicelluloses was filtered off the solid material with suction, and the residue resuspended in water and refiltered three times. Glass wool served as a filter. The combined extracts were neutralized with weak HCl then made barely alkaline with NaOH. Activity was determined on aliquots of these solutions.

Radioactivity in the samples was determined by a solid-counting technique. Because of the high specific activity of the acetate used it was possible to count each sample at infinite thinness. The planchets were counted in a windowless flow counter.

Activities of the various groups of compounds in the auxin-treatments except for the alcohol extracts have been calculated on the basis of an absorption of carbon-14 equal to that of the respective non-auxin-treated controls. In his work with pea stem slices, Perlis (9) found that the alcohol extracts reflect relative rates of C^{14}-absorption in a given pair of experimental treatments with an accuracy of about 5 %. For this reason and because determination of the C^{14}-absorption by difference is not feasible for experiments of 30 minutes and less where only 10 to 15 %

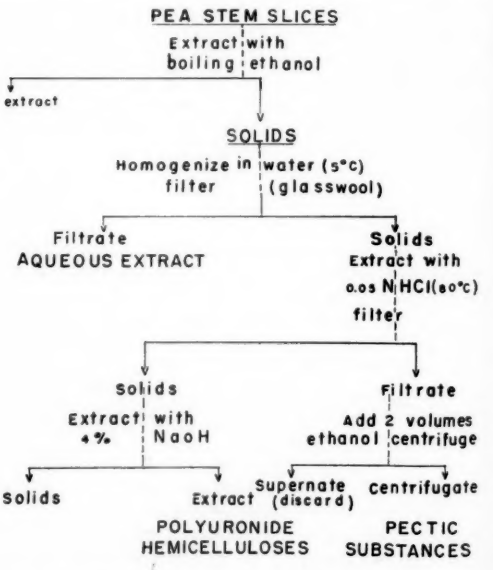

FIG. 2. Procedure for fractionation of pea stem slices. Separation of cell wall constituents.

of the supplied isotope is taken up by the tissues, the alcohol extracts have been used as measures of relative C^{14}-absorption. One exception to this general rule is to be found in the data of table IV. Because two treatments of this experiment (7 and 8) were supplied with a much higher level of acetate-1-C^{14} than the others, results of these two treatments are calculated on the basis of the alcohol extract of a control given the lower level of IAA (treatment 5).

Results

Effects of IAA on the utilization of acetate-1-C^{14} by pea stem slices in four hours are shown in table I. In view of the inhibitory effects of calcium on growth and the probability that these effects involve the pectic substances it appeared that inclusion of a calcium salt in the test solutions might accentuate effects of auxin on this class of compounds. All treatments of this experiment include $CaCl_2$ at 0.005 M. Acetate-1-C^{14} was supplied as the calcium salt. Addition of IAA, adjusted to pH 4.7 with $Ca(OH)_2$, to the basal solution enhances growth and the incorporation of carbon-14 into the organic acids and CO_2 appreciably, inhibits incorporation into the lipides, and has insignificant effects on syntheses of other groups of compounds assayed.

Addition of KCl to the $CaCl_2$-Ca-acetate mixture produces small increases in growth and incorporation of acetate into organic acids. When IAA is added along with the KCl the growth increment is very appreciably enhanced but the only effects of possible significance are increases in the activities of the respiratory CO_2 and the lipides. For the last experimental treatment the IAA concentration was raised to 2.5×10^{-4} M, with the other salts as in the previous treatment. This level of IAA, obviously quite high, more than doubles the growth rate of the slices, though the growth promotion is less than that elicited by the lower level of auxin. Absorption of carbon-14, as revealed by the activity of the alcohol extract, is somewhat greater than that for the lower level of IAA. The most impressive effect of the higher level of auxin is that it inhibits incorporation of carbon-14 into the pectic substances. An inhibitory effect on the formation of $C^{14}O_2$ of about 28 % may be of significance.

These results are, in their rather unimpressive nature, quite similar to those obtained by Perlis (9). Search for a more revealing technique led to the experiment summarized in table II. This and subsequent experiments involve incubation of the slices in a variety of non-radioactive solutions prior to the exposure of the slices to acetate-1-C^{14}. In this experiment the slices were pre-treated for 225 minutes in acetate only, in acetate plus IAA at 2.5×10^{-5} M and in acetate plus IAA at 2.5×10^{-4} M. These three samples and three not given the pre-treatment were then incubated for 30 minutes in acetate-1-C^{14} with each batch of slices exposed to the level of IAA it received in the pre-treatment.

Both levels of IAA promote growth (table II), the higher level having the greater effect in this experiment. Growth for the 30 minutes when they were in the radioactive solutions is reported for the slices not given the pre-treatment. For the other samples, growth occurring in the pre-treatment period is reported.

There is an impressive response to IAA in the pre-treated tissues so far as utilization of acetate-1-C^{14} is concerned. Incorporation of activity into the organic acids is more than doubled at the higher level of IAA, and it is increased by about 50 % at the lower concentration. Incorporation of activity into the pectic substances and to a lesser degree into the water extractable materials is affected by both concentrations. Inhibited conversions of acetate to hemicelluloses, lipides, and CO_2 are produced by the higher level of auxin. The lower level, however, enhances these conversions.

Effects of IAA on the untreated pea stem slices in 30 minutes are not as dramatic as those in the pre-treated tissues, though some appear to be of significance Incorporations into pectic substances, water extractable materials, organic acids, and CO_2 are enhanced appreciably by both levels of auxin Other effects seem to be of doubtful significance

In another experiment the effects of pre-treatment time were considered (table III) For this experiment

Table I

Incorporation of C^{14} from Calcium Acetate-1-C^{14} by Pea Stem Slices in 4 hr. Total Activities. $\times 10^4$ Cpm *

No.	KCl mM/l	IAA μm/l	Fresh wt % gain	Alcohol extract	Pectic subst.	Lipides	Water extract	Organic acids	CO_2
1	0	0	9	5,200	324	160	150	376	854
2	0	25	23	4,790	323	122	151	563	1,100
3	10	0	11	5,290	325	159	148	442	906
4	10	25	33	5,700	359	187	157	479	1,100
5	10	250	29	5,124	206	173	180	437	794

Each treatment included 7 g of pea stem slices which were incubated for 4 hr at 25° C in 100 ml of a basal solution containing $(CH_3C^{14}OO)_2Ca$, 6×10^{-5} M; $CaCl_2$, 5×10^{-3} M; and having 0.54 μc of activity per ml. Each solution was adjusted to pH 4.7 with $Ca(OH)_2$ (No. 3), or KOH (Nos. 4 and 5) or HCl (Nos. 1 and 2).

* Values for the pectic substances, lipides, water extract, organic acids and CO_2 for each IAA-treated sample have been calculated on the basis C^{14}-absorption equal to that of the appropriate control. Activities of the alcohol extracts serve as measures of the relative amounts of C^{14} absorbed.

TABLE II

UTILIZATION OF ACETATE-1-C^{14} BY PEA STEM SLICES IN 30 MIN. EFFECTS OF PRE-TREATMENT WITH UNLABELED ACETATE AND IAA. TOTAL ACTIVITIES. $\times 10^3$ CPM *

No.	PRE-TREATMENT	IAA μm/l	FRESH WT % GAIN	ALCOHOL EXTRACT	PECTIC SUBST.	HEMI-CELLULOSE	WATER EXTRACT	LIPIDES	ORGANIC ACIDS	CO_2
1	None	0	3	5,170	280	220	154	1,130	1,100	1,090
2	None	25	4	4,780	320	239	279	898	1,180	1,570
3	None	250	5	5,790	364	220	225	1,097	1,460	1,260
4	KAc	0	7	6,300	320	190	346	809	1,120	871
5	KAc, IAA	25	25	6,960	248	232	272	1,010	3,290	945
6	KAc, IAA	250	27	7,660	120	129	244	648	4,690	707

For the pre-treatment 4.5 g samples of pea stem slices were incubated in 200 ml of solution containing CH_3COOK, 4×10^{-4} M and IAA as indicated above for 3.5 hr at 24° C.

For the carbon 14-treatment the slices were incubated for 30 min in 50 ml of a solution containing 1×10^{-4} M $CH_3C^{14}OOK$, IAA as indicated above, and 0.9 μc of activity per ml. All solutions were adjusted to pH 4.7 with KOH or H_2SO_4.

* Values for pectic substances, hemicelluloses, water extract, lipides, organic acids and CO_2 for the IAA-treated tissues have been calculated on the basis of C^{14}-absorption equal to that of the appropriate control. Values for the alcohol extracts serve as measures of the relative C^{14}-absorption.

only one level of IAA was employed, 25×10^{-4} M, and this promoted growth in each of the pre-treatment periods Exposure of the slices to acetate-1-C^{14} was limited to 15 minutes.

Most impressive is the inhibition of incorporation into the pectic substances. This effect becomes progressively greater as the pre-treatment periods are extended and eventually activity in the control tissues exceeds that of the IAA-treated by more than 6 fold As with other entities considered here incorporation of activity into the pectic substances of the control tissues shows a similar trend suggesting that the endogenous auxin of the slices affects their activities in a manner like that of the exogenously supplied IAA. Incorporation of activity into hemicelluloses, water extractable materials, and lipides is inhibited to a very appreciable degree by pre-treatment with IAA. Activities in the organic acids and in the CO_2, except for the longest pre-treatment period, are much enhanced by the auxin. Each of these effects is clearly discernible in the tissues that have received only 15 minutes of pre-treatment. As in the previous experiment, certain effects seen in the tissues not given the pre-treatment are reversed in the pre-treated tissues. This is true for pectic substances and organic acids but not for the hemicelluloses, lipides and CO_2.

In another experiment effects of pre-treatments with calcium acetate, $CaCl_2$ and KCl are compared (table IV). Indoleacetic acid is present at a concentration of 5×10^{-4} M in this experiment, and even at this high level promotes growth. It is seen, however, that this level of auxin inhibits absorption of radioactive acetate as indicated by the values for the alcohol extracts. These inhibitions range from 23 % for the KCl pre-treatment to 32 and 35 % for the calcium acetate and $CaCl_2$ treatments respectively. Auxin-inhibition of incorporation of activity into the alcohol extract, hemicelluloses, and water-extractable

TABLE III

UTILIZATION OF ACETATE-1-C^{14} BY PEA STEM SLICES IN 15 MIN. EFFECT OF PRE-TREATMENT TIME. TOTAL ACTIVITIES. $\times 10^3$ CPM *

No.	PRE-TREATMENT MIN	IAA μm/l	FRESH WT % GAIN	ALCOHOL EXTRACT	PECTIC SUBST.	HEMI-CELLULOSE	WATER EXTRACT	LIPIDES	ORGANIC ACIDS	CO_2
1	0	0	..	2,430	149	180	378	142	256	286
2	0	250	..	2,690	164	169	294	156	221	445
3	15	0	9	2,400	131	190	429	204	435	235
4	15	250	12	3,070	95	129	320	136	679	295
5	105	0	10	4,270	88	218	442	169	1,250	120
6	105	250	23	4,730	37	128	234	99	2,120	149
7	225	0	10	4,670	64	177	410	79	965	74
8	225	250	25	5,250	7	92	78	29	1,540	66

For the pre-treatment 3.25 g of pea stem slices were incubated in 200 ml of a solution of CH_3COOK, 4×10^{-4} M and pH 4.7, for the periods indicated.

For the C^{14}-treatment the samples were transferred to 50 ml of a solution containing 1×10^{-4} M $CH_3C^{14}OOK$, adjusted to pH 4.7 with H_2SO_4 and having 0.9 μc of activity per ml.

Concentrations of IAA indicated apply to both treatments.

* Values for the pectic substances, hemicelluloses, water extract, lipides, organic acids and CO_2 for the IAA-treated tissues have been calculated on the basis of a C^{14}-absorption equal to that for the appropriate control tissues. Activities of the alcohol extracts serve as measures of the relative C^{14}-absorption.

TABLE IV

UTILIZATION OF ACETATE-1-C^{14} BY PEA STEM SLICES IN 15 MIN. EFFECTS OF VARIOUS PRE-TREATMENT SOLUTIONS. TOTAL ACTIVITIES. $\times 10^3$ CPM*

No.	PRE-TREATMENT	IAA μm/l	FRESH WT. % GAIN	ALCOHOL EXTRACT	PECTIC SUBST.	HEMI-CELLULOSE	WATER EXTRACT	LIPIDES	ORGANIC ACIDS	CO_2
1	None	0	..	1,360	68	79	426	313	157	984
2	None	500	..	1,360	81	101	265	338	176	646
3	$Ca(Ac)_2$	0	6	1,470	13	15	88	52	309	169
4	$Ca(Ac)_2$	500	24	1,000	9	2	24	38	389	584
5	$CaCl_2$	0	7	1,980	62	51	230	343	271	205
6	$CaCl_2$	500	20	1,290	7	9	46	105	581	688
7	KCl	0	11	5,070	41	49	207	173	303	30
8	KCl	500	27	3,900	6	68	61	118	371	40

For pre-treatment 3.75 g of pea stem slices were incubated 225 min at 24° C in 200 ml of $(CH_3COO)_2Ca$, $CaCl_2$ or KCl with IAA as indicated above. Each salt was used at 5×10^{-4} M and each solution was adjusted to pH 4.7 with $Ca(OH)_2$ (1 through 6), KOH (7 and 8), and HCl as needed.

For carbon 14-treatments 1 through 6 the slices were incubated 15 min in 50 ml of a solution containing $(CH_3C^{14}OO)_2Ca$, 6×10^{-5} M, with IAA as indicated above and the pH adjusted to 4.7 as for the pre-treatment solutions, and having 0.54 μc/ml of activity. Solutions for C^{14}-treatments 7 and 8 differed in that $CH_3C^{14}OOK$ was used at 1×10^{-4} M, 0.9 μc/ml.

* Values for pectic substances, water extract, hemicelluloses, lipides, organic acids and CO_2 of the IAA-treated tissues and No. 7 have been calculated on the basis of C^{14}-absorption equal to that of the appropriate control. Treatments 1 and 2 are controls for 2 and 4 respectively, 5 is the control for 6, 7 and 8. Activities of the alcohol extracts serve as measures of the relative C^{14}-absorption.

materials is significantly greater in the tissues pre-treated with calcium acetate than it is in those given $CaCl_2$. It may be of greater importance, however, that the acetate ion itself profoundly inhibits syntheses of pectic substances, hemicelluloses, water-extractable materials and lipides. Enhancement of organic acid synthesis by IAA is greater in the $CaCl_2$ treatment, and the effect on the activity in the CO_2 is about equal in the two calcium pre-treatments. Perhaps the most interesting difference between the calcium pre-treatments and that with KCl relates to activity in the respiratory CO_2. There is much less activity in the CO_2 produced by the KCl-treated tissues, and the enhancement of CO_2 formation from the acetate by auxin is far less for these tissues.

Auxin-effects on the slices used without pre-treatment differ from those of the previous experiment (table III) so far as two entities are concerned, organic acids and respiratory CO_2. In the experiment of table III, where the added cation is potassium, IAA inhibits incorporation into organic acids and enhances it for CO_2. In the experiment of table IV where calcium is the added cation the reverse occurs.

Effects of pre-treatment in KCl or $CaCl_2$ alone followed by exposure to radioactive acetate with and without IAA were determined in an experiment summarized in table V. No dramatic effects are to be seen in this experiment though some of the differences in activity appear to be significant. Absorption of acetate-1-C^{14} is appreciably increased by IAA in the tissues pre-treated with KCl, but this is not true for the $CaCl_2$-treated slices. Contrary to the effect one obtains when untreated slices are used, there is here a small inhibition of incorporation into the pectic substances by IAA. Inhibitory effects on lipide synthesis are of a similar order of magnitude. Incorpo-

TABLE V

UTILIZATION OF ACETATE-1-C^{14} BY PEA STEM SLICES IN 15 MIN. EFFECTS OF IAA AFTER PRE-TREATMENT WITH KCl OR $CaCl_2$ ONLY. TOTAL ACTIVITIES. $\times 10^3$ CPM *

No.	PRE-TREATMENT	IAA μm/l	ALCOHOL EXTRACT	PECTIC SUBST.	WATER EXTRACT	LIPIDES	ORGANIC ACIDS	CO_2
1	KCl	0	4,580	52	302	788	2,610	289
2		250	6,330	48	239	671	3,380	256
3	$CaCl_2$	0	6,480	92	387	860	4,130	253
4		250	6,750	78	369	780	3,690	219

For pre-treatment 4 g of pea slices were incubated at 26° C for 225 min in 100 ml of KCl or $CaCl_2$ at 5×10^{-4} M.

For the C^{14}-treatment the tissues were incubated for 15 min at 26° C in 50 ml of a solution containing $CH_3C^{14}OOK$ at 1×10^{-4} M, having 0.9 μc/ml of activity and adjusted to pH 4.7 with HCl.

* Values for pectic substances, water extract, lipides, organic acids and CO_2 of the IAA-treated tissues have been calculated on the basis of C^{14}-absorption equal to that of the appropriate control. Activities of the alcohol extracts serve as measures of the relative C^{14}-absorption.

ration of C^{14} into the organic acids is enhanced by IAA in the KCl treatment (30 %) and inhibited in the $CaCl_2$ treatment (11 %).

Discussion

It is apparent from the pre-treatment studies that the utilization of acetate by pea stem slices is profoundly influenced by IAA. Two aspects of these experiments suggest reasons for the failure of studies involving simultaneous exposure to acetate-1-C^{14} and IAA to reveal similar differences.

The first of these relates to the fact that in a number of the groups of compounds analyzed the responses to auxin in the 1st period of 15 minutes are the opposite of those observed in the 2nd period. This is true, for example, for experiment III with respect to incorporation in pectic substances, lipides, and organic acids. Incorporation for each is promoted in the 1st period and inhibited for each in the 2nd period after 15 minutes of pre-treatment with cold acetate and IAA. With simultaneous exposure to IAA and radioactive acetate one obtains an average of these opposing effects resulting in the appearance of no effect or the occurrence of a smaller one.

Secondly, it is well known that prolonged exposure to labeled metabolites commonly leads to a random distribution of the label. This effect is clearly indicated in tables II and III, in which results of exposures to acetate-1-C^{14} of 30 and 15 minutes are reported. Appreciably smaller experimental effects are produced by the longer incubation in the labeled material. An even more random distribution appears to be characteristic of the experiment in which the tissues were in the radioactive solution for four hours (table I). As the label becomes more randomly distributed, syntheses of cellular constituents by processes not affected by auxin mask those that are.

It may be stated as a general summary of this study that IAA inhibits the incorporation of radioactivity from acetate-1-C^{14} into pectic substances, hemicelluloses and lipides, but enhances incorporation into organic acids and CO_2. Competition between synthetic pathways utilizing the acetate appears to be concerned. Exogenously supplied IAA augments the rate at which acetate is drawn into pathways of acid metabolism and probably in this manner reduces the availability of the labeled substrate for syntheses of lipides and polysaccharides.

Summary

Two experimental techniques have been used in a study of the effects of IAA on the utilization of acetate-1-C^{14} by pea stem slices. The first and less revealing technique involves the simultaneous presentation of IAA and acetate-1-C^{14} to the tissues. In slices so treated for four hours with calcium as the sole basic cation, IAA (2.5×10^{-5} M) stimulates incorporation of activity into respiratory CO_2 and organic acids and inhibits it for lipides. With the addition of KCl incorporation into the lipides is stimulated to a small degree but the other results are not appreciably different. With KCl and IAA at 2.5×10^{-4} M incorporation into the pectic substances is inhibited. A greater part of the study is concerned with the effects of pre-treatment with unlabeled potassium acetate or other salts plus and minus IAA, followed by brief exposure of the tissue to acetate-1-C^{14}. A typical response to 105 minutes of pre-treatment and 15 minutes of C^{14}-treatment is a strong inhibition of incorporation into pectic substances, lipides and water extractable materials and a marked stimulation of incorporation into organic acids, and respiratory CO_2 by IAA at a growth-promoting concentration of 2.5×10^{-4} M. In the presence of potassium salts the effect of IAA is much greater on organic acids than on CO_2; with calcium the effect of IAA is about the same on these two entities. It appears that IAA promotes entry of acetate into pathways of organic acid metabolism and thereby reduces the availability of this substrate to other systems.

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THE RELATIONSHIP OF MAGNESIUM ION AND MOLECULAR STRUCTURE OF 2,4-DICHLOROPHENOXYACETIC ACID AND SOME RELATED COMPOUNDS TO THE INHIBITION OF THE RESPIRATION OF AZOTOBACTER VINELANDII[1]

EMMETT J. JOHNSON[2,3] AND ARTHUR R. COLMER

DEPARTMENT OF BOTANY, BACTERIOLOGY AND PLANT PATHOLOGY AND
LOUISIANA AGRICULTURAL EXPERIMENT STATION, LOUISIANA STATE UNIVERSITY,
BATON ROUGE, LOUISIANA

The independent work of Heath and Clark (1, 2) and Johnson and Colmer (3, 4, 5) introduced a new concept concerning the mode of action of auxin and auxin-like substances. Their evidence suggested interference with metal ion metabolism of the organisms studied. The concept would seem to be more compelling in light of the fact that the two pairs of workers using entirely different approaches, one the measurement of plant growth induction and the other the inhibition of bacterial respiration, arrived at the same conclusion.

The argument becomes still more convincing in view of the fact that in one instance the natural auxin indoleacetic acid (IAA) was used and in the other the synthetic auxin-like substance 2,4-dichlorophenoxyacetic acid (2,4-D) was the test compound. Of still further interest are the facts that in both instances a parallel was established between the action of the test substance and known chelating compounds, and, as shown in the latest work of Johnson and Colmer (5), that the inhibitions produced by 2,4-D, aureomycin, and achromycin were all abolished by low molar ratios of magnesium to the test agents.

In view of these new findings it seemed advisable to investigate the relationship between structural specificity and activity of 2,4-D and related compounds to determine whether or not there was a correlation with the magnesium content of the external environment.

MATERIALS AND METHODS

The organism used was *Azotobacter vinelandii* strain O. The N-free mineral salts solution of Burk (Wilson and Knight (7)) with 1.0% mannitol was the growth medium. The organisms were grown in 300 ml of medium in a 1,000-ml flask on a rotary-type shaker at 30° C for 16 to 18 hours.

At the end of the growth period the cells were collected by centrifugation at 4° C. The cells were washed and centrifuged once with 10 ml of 0.2% aqueous KCl solution, pH 6.9. They were suspended in 0.2% KCl and filtered through Reeve Angel filter paper no. 202, and the resulting suspension was adjusted to an optical density of 1.0 with a B and L Spectronic 20 spectrophotometer at 420 mμ.

The usual Warburg procedures were employed (6). Each flask had in the reaction chamber the following materials expressed as final molar concentrations in a total volume of 2.5 ml: cells, 0.5 ml; phosphate buffer pH 6.9 to 7.0, 0.08 M; $MgSO_4$, and the test compounds as indicated. The side arms of the flasks contained 0.1 ml of a 0.1 M solution of mannitol which when tipped gave a final concentration of 0.004 M. The center well contained 0.1 ml of 10% KOH and a fluted filter paper strip. All vessels were made to volume with triple distilled water.

The gas phase was air and the temperature was 31°C. The vessels were allowed to equilibrate for 5 minutes and then the substrate was tipped in from the side arms. Oxygen uptake was measured for 60 minutes. The 2,4-D used was obtained from the Dow Chemical Co. and was labeled "pure." All of the other compounds were purchased from the Eastman Kodak Co.

RESULTS AND DISCUSSION

Table I shows that 2,4-D alone produced complete inhibition of the respiration of *A. vinelandii* at the concentration of 9.2×10^{-3} M. Neither of the single chlorine-bearing compounds was as effective at this concentration as was 2,4-D, but *para*-chlorophenoxyacetic acid was the more effective of the single chlorine substituted derivatives of phenoxyacetic acid. Although *ortho*-chlorophenoxyacetic acid produced less inhibition than *para*-chlorophenoxyacetic acid, it was more toxic than phenoxyacetic acid. This latter compound, without any chlorine in the molecule, was least effective of all compounds tested. Phenylpropionic acid, which is without the oxygen atom of the phenoxy compounds but which has the

TABLE I

INHIBITION OF THE RESPIRATION OF A. VINELANDII BY 2,4-D AND SOME RELATED COMPOUNDS

TEST SYSTEM	μL O_2 UPTAKE IN 60 MINUTES
Active control	288 *
9.2×10^{-3} M 2,4-D	0
9.2×10^{-3} M *para*-chlorophenoxyacetic acid	81
9.2×10^{-3} M *ortho*-chlorophenoxyacetic acid	173
9.2×10^{-3} M phenylpropionic acid	202
9.2×10^{-3} M phenoxyacetic acid	244

* Values reported in all tables are averages of duplicate vessels. Each experiment was run at least twice to assure reproducibility of the results reported.

[1] Received August 8, 1957.

[2] Present address: Department of Medical Microbiology, Stanford University, Stanford, Calif.

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TABLE II

RELATIONSHIP BETWEEN PHENOXYACETIC ACID AND MAGNESIUM IN THE RESPIRATION OF A. VINELANDII

TEST SYSTEM	μL O_2 UPTAKE IN 60 MINUTES
Active control	283
2×10^{-2} M phenoxyacetic acid	183
2×10^{-2} M phenoxyacetic acid + 1×10^{-2} M $MgSO_4$	257
2×10^{-2} M phenoxyacetic acid + 2×10^{-2} M $MgSO_4$	287
2×10^{-2} M phenoxyacetic acid + 4×10^{-2} M $MgSO_4$	316

length of its side chain maintained by substitution of a CH_2 group for the oxygen atom, was less inhibitory than either of the chlorine substituted derivatives, but was more inhibitory than phenoxyacetic acid. These findings are in agreement with those of Zimmerman and Hitchcock (8) in their work with higher plants.

Before the relation of magnesium to the toxicity of the test agents could be determined, the concentration of each compound which would markedly affect the respiration of the organism, without complete inhibition, had to be ascertained. The concentration chosen for use with both the *ortho-* and *para*-chlorophenoxyacetic acids was that concentration which in the preliminary experiments was just prior to the concentration which had produced complete inhibition. In the case of phenylpropionic and phenoxyacetic acids, solubility limitations prevented the use of any higher concentration than 2×10^{-2} M.

Tables II to V give the results of the tests on the effect of added magnesium upon the toxicity of the compounds. Protection from the inhibition of respiration produced by phenoxyacetic acid at 2×10^{-2} M was provided by the addition of magnesium at a 1 : 1 molar ratio (table II). With 2×10^{-2} M phenylpropionic acid, the inhibition was prevented by a molar ratio of 2 : 1, magnesium to the test compound (table III). *Ortho*-chlorophenoxyacetic acid at 2×10^{-2} M almost completely inhibited oxygen uptake. A molar ratio of 2 : 1, magnesium to the test compound provided considerable protection (table IV), although not complete protection at this molar ratio. Higher molar ratios were not tested.

TABLE III

RELATIONSHIP BETWEEN PHENYLPROPIONIC ACID AND MAGNESIUM IN THE RESPIRATION OF A. VINELANDII

TEST SYSTEM	μL O_2 UPTAKE IN 60 MINUTES
Active control	398
2×10^{-2} M phenylpropionic acid	163
2×10^{-2} M phenylpropionic acid + 1×10^{-2} M $MgSO_4$	330
2×10^{-2} M phenylpropionic acid + 2×10^{-2} M $MgSO_4$	356
2×10^{-2} M phenylpropionic acid + 4×10^{-2} M $MgSO_4$	398

TABLE IV

RELATIONSHIP BETWEEN ORTHO-CHLOROPHENOXYACETIC ACID AND MAGNESIUM IN THE RESPIRATION OF A. VINELANDII

TEST SYSTEM	μL O_2 UPTAKE IN 60 MINUTES
Active control	305
2×10^{-2} M *ortho*-chlorophenoxyacetic acid	20
2×10^{-2} M *ortho*-chlorophenoxyacetic acid + 1×10^{-2} M $MgSO_4$	158
2×10^{-2} M *ortho*-chlorophenoxyacetic acid + 2×10^{-2} M $MgSO_4$	194
2×10^{-2} M *ortho*-chlorophenoxyacetic acid + 4×10^{-2} M $MgSO_4$	266

Para-chlorophenoxyacetic acid strongly inhibited oxygen uptake at a concentration as low as 8×10^{-3} M and protection from this degree of inhibition (table V) was afforded by magnesium at a 1 : 1 molar ratio.

The fact that the inhibition by 2,4-D (5) could be prevented by a molar ratio of 1 : 1 magnesium to the herbicide and that all of the compounds tested here could also have their toxicity abolished by low molar ratios of magnesium, lends added support to the contention that their mode of action involves the interference with metal ion metabolism. The data presented here also suggest that the inhibition by 2,4-D of the respiration of *A. vinelandii* is a function of the whole molecule rather than any one part. Of the parts of the molecule that would seem to be the most likely sites for binding of metal ions, in addition to the carboxyl group of the acetic acid moiety, the chlorine atoms seem to be the most significant substituents and the oxygen atom of the phenoxy moiety seems to be the least significant.

TABLE V

RELATIONSHIP BETWEEN PARA-CHLOROPHENOXYACETIC ACID AND MAGNESIUM IN THE RESPIRATION OF A. VINELANDII

TEST SYSTEM	μL O_2 UPTAKE IN 60 MINUTES
Active control	317
8×10^{-3} M *para*-chlorophenoxyacetic acid	80
8×10^{-3} M *para*-chlorophenoxyacetic acid + 4×10^{-3} M $MgSO_4$	228
8×10^{-3} M *para*-chlorophenoxyacetic acid + 8×10^{-3} M $MgSO_4$	290
8×10^{-3} M *para*-chlorophenoxyacetic acid + 16×10^{-3} M $MgSO_4$	335

SUMMARY

At a concentration of 9.2×10^{-3} M only 2,4-D completely inhibited the respiration of *Azotobacter*

vinelandii. The related compounds tested, phenoxyacetic acid, phenylpropionic acid, *ortho-* and *para*-chlorophenoxyacetic acids, inhibited the test organism to lesser degrees.

Protection from the inhibition produced by 2×10^{-2} M phenoxyacetic acid and phenylpropionic acid was afforded by molar ratios of 1 : 1 and 2 : 1 respectively, magnesium to the agents.

Considerable protection, though not complete protection, from the inhibition produced by 2×10^{-2} M *ortho*-chlorophenoxyacetic acid was provided by a molar ratio of 2 : 1, magnesium to the test compound.

A 1 : 1 molar ratio of magnesium to the agent provided protection from the inhibition produced by *para*-chlorophenoxyacetic acid at 8×10^{-3} M.

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AN EFFECT OF GIBBERELLIC ACID ON THE FLOWERING OF XANTHIUM, A SHORT DAY PLANT[1,2]

R. G. LINCOLN and K. C. HAMNER

Division of Natural Science, Long Beach State College, Long Beach, California and Department of Botany, University of California at Los Angeles, Los Angeles, California

A single leaf detached from a flowering Xanthium plant if grafted to a vegetative plant will initiate flowering in the second plant (10). From this and other evidence, the concept that leaf tissue is the source of the flowering stimulus is well established.

Carr (2, 3) debudded plants prior to short day treatment and found the mature leaves of Xanthium to be incapable of supplying stimulus to a vegetative bud that was grafted to the debudded stock immediately following the post-inductive treatment. Debudded plants can, however, serve as a source of stimulus to a subsequently grafted bud if immature leaves are present on the debudded plant at the time of the short day treatment (6). The role of immature tissue in the flowering reaction is further emphasized by evidence that in the absence of young leaves the flowering response of a single receptor bud is correlated with the growth activity of that bud at the time of photoinductive treatment (6).

Meristematic tissues have been recognized as centers of synthesis for auxin and more recently immature tissues of the seed and stem have been associated with "gibberellin-like" substances (8, 9, 11, 12). Auxins stimulate flowering in certain long day plants maintained on conditions of photoperiod very near the threshold of flowering (7). Salisbury (13) has demonstrated a significant increase in the flowering response of a slow growing bud of Xanthium following applications of auxin. Long day conditions in plants requiring such treatment for floral initiation as well as the cold requirement for biennial plants can in many instances be replaced by gibberellin (4). At least one plant of the long-short day type has been caused to flower by applications of gibberellin (1). In view of the association of "gibberellin-like" substances with immature tissues the following series of experiments was performed to test for a possible relationship of gibberellin to the previously mentioned promotion of flowering by the young leaf and bud tissues of Xanthium.

Materials and Methods

The plant material utilized in the experiments reported in this paper was prepared by planting fruit of cocklebur, *Xanthium pennsylvanicum*, which had been collected from the vicinity of Chicago, Illinois. The burs were planted in shallow flats of soil maintained in the UCLA Botany Greenhouse. About four days after emergence, the seedlings were transplanted to 4-inch pots. With the exception of the

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[2] The gibberellic acid, sample number 56R3543, used in this study was supplied by the Eli Lilly Company through the courtesy of Dr. Curt Leben.

actual interval of inductive treatment all plants were maintained on long-day conditions throughout their growth period. Short-day conditions were achieved by placing the plants on a greenhouse bench which was covered daily from 5:00 PM until 8:00 AM of the following day with an opaque curtain. Gibberellic acid, henceforth referred to as GA_3 (14), was applied dropwise with a hypodermic syringe to the leaf surface or in the axils of the leaves, using 100 μg in 0.1 ml of water per plant per treatment. Care was taken in debudding to excise each bud completely to preclude regeneration. Ten plants were employed for each treatment.

Results were recorded employing a numerical evaluation based on the size and stage of development of the terminal staminate inflorescence at some specified time following inductive treatment. Vegetative plants were rated as zero on the scale. The smallest recognizable flowering apex was assigned a value of 0.1. An apex of floral primordia measuring one fourth millimeter in diameter was evaluated as 0.2. An additional increment of 0.1 was allowed for every successive one fourth millimeter increase in the diameter of the developing inflorescence. Each value expressed in tables I, II and III is the sum of the individual ratings of the flowering development of 10 plants comprising a single treatment. The details of this system of evaluation are given in a previous paper (5).

The effect of GA_3 on the flowering response of Xanthium was tested in three experiments: a) The flowering response of a slow growing cotyledonary bud; b) The flowering potential of debudded plants; and c) The flowering response of intact plants.

Experiments and Results

Effect of GA_3 on the Flowering Response of a Slow Growing Bud: Under optimum conditions Xanthium grows with an unbranched single stalk habit. The growth activity of cotyledonary buds and leaf axillary buds is normally suppressed by the dominance of the stem tip. Severing the stem within a mature internode and excising all buds in the axils of those leaves above the cotyledons stimulates the growth of the cotyledonary buds. For a period of several days the growth of the cotyledonary buds appears to increase in rate as the interval following the debudding treatment is extended. Earlier work (6) indicates that there is a correlation between the relative growth activity of the bud and the level of its response to an inductive treatment. A slow growth rate of the cotyledonary bud at the time of inductive treatment results in a comparatively low flowering response.

The following experiment was performed to determine whether applications of GA_3 could promote flowering in a slow growing bud.

The plants employed in this work, the results of which are cited in table I, were allowed to grow under long-day conditions until one leaf above the primary leaves had achieved full expansion. The plants were then prepared for treatment by first severing the stem in the internode above the primary leaves. The buds in the axils of the primary leaves were then excised and one of the two cotyledonary buds was removed. At the time of inductive treatment the stem supported two primary leaves and a single cotyledonary bud in all treatments with the exception of treatment 5 (table I) which had been completely defoliated. Plants of treatments 1, 2 and 5 had been partially defoliated and debudded, according to the description given in table I, one day before the 1st short day treatment. The plants of treatments 3, 4 and 6 had been prepared for photoinductive treatment on the morning of the 1st short day (3-19-57). GA_3 was administered to treatments 1, 3 and 6 several hours before the 1st dark period. All plants were given two photoinductive cycles and were dissected two weeks following the last short day (4-3-57).

The plants of treatment 6 were completely vegetative indicating that GA_3 applied to Xanthium on long-day conditions will not induce floral initiation. Treatment 5 was also entirely vegetative which confirms earlier results (6) showing that the cotyledonary bud in its early stages of growth will not initiate flowers even though exposed to short day treat-

Table I

Effect of GA_3 on the Flowering Response of Slow Growing Cotyledonary Buds *

Treatment	Magnitude of flowering response and standard error
1. All leaves but two primary leaves removed. Defoliated and debudded one day before the 1st photoinductive cycle. Two short days administered. Treated with GA_3.	8.0 ± 1.60
2. All leaves but two primary leaves removed. Defoliated and debudded one day before the 1st photoinductive cycle. Two short days administered. Not treated with GA_3.	2.8 ± 0.34
3. All leaves but two primary leaves removed. Defoliated and debudded on the day of the 1st photoinductive cycle. Two short days administered. Treated with GA_3.	6.2 ± 1.43
4. All leaves but two primary leaves removed. Defoliated and debudded on the day of the 1st photoinductive cycle. Two short days administered. Not treated with GA_3.	1.7 ± 0.50
5. All leaves removed. Not treated with GA_3. Two short days administered.	0.0
6. All leaves but two primary leaves removed. Treated with GA_3. No short day treatment administered.	0.0

* All buds removed except a single cotyledonary bud. Numerical evaluation of flowering response has been described in a previous paper (5).

ment. The GA_3 treated groups of both series, 1 and 3, exhibited a threefold increase in magnitude of floral development over the comparable untreated series, 2 and 4. The results cited in table I are highly significant. It is apparent that applications of GA_3 increase the flowering response of Xanthium under conditions in which the flowering response would normally be restricted by the slow growth activity of the bud.

INFLUENCE OF GA_3 ON THE EFFECTIVENESS OF SHORT DAY TREATMENT OF DEBUDDED PLANTS: Carr (3) subjected debudded Xanthium plants possessing only mature leaves to short day treatment. He could determine no flowering response in scion buds that were grafted to the debudded stocks subsequent to the short day treatment. A low level of flowering has been demonstrated in the scion bud when young leaves are present on the stock plant at the time the short day treatment has been administered (6). Much higher flowering is achieved when the stock plant is intact at the time of inductive treatment but debudded after the last short day (6).

The following experiment was designed to determine if bud tissue can be replaced during inductive treatment by applications of GA_3.

All buds were removed from 40 Xanthium plants having at least three mature leaves above the primary leaves. Twenty plants retained all leaves up to a leaf about 1.0 cm in length. The remaining 20 plants retained only the mature leaves. All plants were given five photoinductive cycles. GA_3 was applied several hours preceding the 2nd and the 4th dark periods. A vegetative plant was approach grafted to each debudded stock following the last inductive dark period.

The results depicted in table II indicate that GA_3 greatly increases the effectiveness of inductive treatment given to a debudded plant with young leaves. The application of GA_3 to debudded plants having only mature leaves did not result in a flowering response in the scion.

TABLE II

EFFECT OF GA_3 ON DEBUDDED PLANTS GRAFTED TO VEGETATIVE BUDS AFTER INDUCTIVE TREATMENT *

TREATMENT	MAGNITUDE OF FLOWERING RESPONSE AND STANDARD ERROR
I. Mature leaves only. No GA_3	0.0
II. Mature leaves only. With GA_3	0.0
III. Mature leaves + young leaves. No GA_3	3.7 ± 0.88
IV. Mature leaves + young leaves. With GA_3	10.3 ± 1.76

* All plants debudded before inductive treatment. Numerical evaluation of flowering response has been described in a previous paper (5).

EFFECT OF GA_3 ON THE FLOWERING RESPONSE OF INTACT PLANTS: In addition to the greater flowering response associated with the application of GA_3 to plants employed in those experiments cited in tables I and II, the treated plants exhibited a marked acceleration of growth. The following experiment was performed in recognition of the possibility that increased flowering resulting from applications of GA_3 might be attributable to a greater capacity of the bud to respond associated with the observed increased rate of growth.

Three photoinductive series (1, 5 and 10 short days) were used and two treatments of 10 plants each were employed for each series. The plants were maintained intact without debudding or defoliation of any kind. The initial date for each series was staggered so that the last photoinductive cycle for each series was given on the same day (6-27-57). One treatment on each of the three photoinductive series received GA_3. The chemical was applied before the 1st inductive dark period for the treated plants of all three series and also on the morning of the 5th short day for the treated plants on the 10 short day series. All plants were dissected 20 days following the last short day treatment. Although there was very obvious growth acceleration on all treatments which had received GA_3, there was no significant change in the flowering response as a result of applications of the chemical. A similar experiment with Biloxi soybean, subjected to a variety of inductive and non-inductive short day treatments, showed no significant difference in the number of nodes flowering of GA_3 treated plants compared to the flowering response of plants not treated with GA_3. These negative results are in accord with work previously reported by Lang (4).

DISCUSSION

Many plants, annual and biennial, which flower in response to long day have been stimulated to flower under adverse day length conditions following applications of gibberellin. This report constitutes evidence for an increased flowering response of a short day plant attributed to application of GA_3.

This effect can be demonstrated in Xanthium only when the inductive treatment, with coincident application of GA_3, has been administered to plants having a limited amount of immature tissue. In this respect, debudded Xanthium plants having only mature leaves at the time of short day treatment, either with or without GA_3, did not initiate flowering in a scion bud. The flowering stimulus can be demonstrated in debudded plants following short day treatment if young leaves are present (6). This flowering response is greatly intensified if GA_3 is applied (table II). Similarly, if no other buds or young leaves are present, a single slow growing cotyledonary bud will respond only slightly to inductive treatment. Applications of GA_3 at the time of inductive treat-

TABLE III

FLOWERING RESPONSE OF INTACT PLANTS WITH AND WITHOUT GA_3 TREATMENT *

TREATMENT	MAGNITUDE OF FLOWERING RESPONSE AND STANDARD ERROR
I. One photoinductive cycle	
(a) Without GA_3	7.9 ± 0.37
(b) With GA_3**	7.9 ± 0.43
II. Five photoinductive cycles	
(a) Without GA_3	17.4 ± 1.04
(b) With GA_3**	17.8 ± 1.20
III. Ten photoinductive cycles	
(a) Without GA_3	23.2 ± 1.28
(b) With GA_3***	16.8 ± 0.75

* Numerical evaluation of flowering response has been described in a previous paper (5).
** One application of GA_3.
*** Two applications of GA_3.

ment cause a threefold increase in the flowering response (table I).

Intact plants, with a full complement of young leaves and actively growing buds, flower strongly in response to short day treatment. Applications of GA_3 do not alter the flowering response (table III). Such plants may have optimum amounts of the "gibberellin-like" materials since these substances are produced by a variety of immature tissues (9, 11, 12) and may be expected to be present in young leaves and buds. Conversely, other plants that have immature tissue present in limited amounts can be considered to have a low "gibberellin-like" complement. If gibberellin were involved in the flowering response, a plant low in native gibberellin would react strongly to applications of GA_3 as is indicated by the data listed in tables I and II.

Immature tissues apparently constitute the site of GA_3 action in enhancing the flowering of Xanthium as applications of the chemical are not effective in the absence of young leaves or buds. The effect of GA_3 described in these experiments is probably not that of increasing the primary production of the stimulus but may be the result of an increased capacity for the storage of the stimulus in the immature tissues of the young leaves and buds.

SUMMARY

The present experiments constitute evidence for an increase in the flowering response of Xanthium as a result of treatment with GA_3.

GA_3 did not alter the flowering response of intact Xanthium plants. Debudded plants, with only mature leaves remaining, did not serve as a source of the flowering stimulus either with or without GA_3. GA_3 will cause a threefold increase in the flowering potential of debudded plants with young leaves. A similar increase was demonstrated in the flowering response of slow growing cotyledonary buds following application of GA_3.

Immature tissue is considered to be the site of action for GA_3 in promoting the flowering of Xanthium. It is suggested that GA_3 may act by promoting an increased capacity for the storage of the stimulus in the immature tissues.

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THE ASSIMILATION OF N^{15}-FROM LABELED HYPONITRITE BY SOYBEAN LEAVES[1,2]

D. STUART FREAR[3] AND R. C. BURRELL

DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, THE OHIO STATE UNIVERSITY, COLUMBUS 10, OHIO AND THE CHARLES F. KETTERING FOUNDATION, YELLOW SPRINGS, OHIO

Hyponitrite has often been suggested as a possible intermediate between nitrite and hydroxylamine in the stepwise reduction of nitrate to ammonia by higher plants. It has only been very recently, however, that direct evidence has become available in support of this hypothesis through the discovery and partial characterization of nitrate, nitrite and hydroxylamine reductase enzyme systems in plants. The existence of an intermediate in plants at the reduction level of hyponitrite has been indirectly indicated by recent findings. Nason, Abraham and Averbach (16) demonstrated that three moles of reduced pyridine nucleotide are necessary for the reduction of one mole of nitrite to ammonia in plant extracts. In studying the stoichiometry of hydroxylamine reductase systems in plant extracts, Zucker and Nason (31, 32) found that one mole of reduced pyridine nucleotide was necessary for the reduction of one mole of hydroxylamine to ammonia. These facts, coupled with our present knowledge of hydrogen transfer mechanisms in biological systems, strongly suggest that there is an intermediate with an oxidation state of + 1 for nitrogen between nitrite and hydroxylamine in the reduction of nitrate to ammonia by two-electron steps.

Hyponitrite has never been detected in higher plants and, until recently, hyponitrite had never been shown to be utilized by plants. In the recent reports of Medina and Nicholas (12, 13), however, the authors demonstrated the formation of ammonia from hyponitrite by extracts of Neurospora.

The objective of the investigations reported here is to ascertain, through the use of N^{15}-labeled hyponitrite, whether or not hyponitrite-nitrogen is assimilated by higher plants.

MATERIALS AND METHODS

PREPARATION OF N^{15}-LABELED SODIUM HYPONITRITE: Labeled nitric acid (34.5 atom percent N^{15}) was obtained from Distillation Products Industries and converted to sodium nitrate prior to a direct reduction to hyponitrite with sodium amalgam. The resulting labeled sodium hyponitrite preparation was stored in a vacuum desiccator at room temperature. The procedures used were essentially those of Divers (6, 7, 8) as modified by Partington and Shah (22) and Addison, Gamlin and Thompson (2).

DETERMINATION OF HYPONITRITE PURITY: The volumetric procedure of Oza (19) for the determination of hyponitrite by the titration of the insoluble silver salt with standard potassium thiocyanate solution was slightly modified so that as little as 10 micromoles of hyponitrite could be determined in the presence of nitrate and nitrite. Nitrate impurities were determined by the procedure of Mellette, Brodsky and Palmer (14) after the removal of the hyponitrite by boiling (19, 20). Nitrite impurities were determined by the method of Shinn (24). Hydroxylamine and ammonia impurities were tested for by the procedures of Frear and Burrell (11) and with Nessler's reagent respectively.

INTRODUCTION OF LABELED HYPONITRITE: Four grams of fresh, primary leaves from 8-day-old soybean seedlings were harvested, rinsed with distilled water and cut into small sections approximately 5 mm square. The leaf sections were immediately placed in a 125-ml suction flask and vacuum infiltrated (30) with 25 ml of 0.01 M labeled sodium hyponitrite solution at pH 11.3. Infiltration was carried out in diffuse light with intermittent breaking of the vacuum and continuous shaking for 10 minutes. After infiltration, the remaining labeled sodium hyponitrite solution was quickly drained off and the infiltrated leaves were rinsed with distilled water. The infiltrated leaf sections were then divided into two equal samples and spread out on 6-inch watch glasses. One of the samples was exposed to light for 60 minutes (approximately 1,000 ft-c intensity) and the other was maintained in darkness for 60 minutes. Another 4-g portion of leaf sections was infiltrated with a previously boiled 0.01 M labeled sodium hyponitrite solution, divided into two equal samples, and run simultaneously in the light and in the dark as a control for any N^{15}-labeled impurities or decomposition products in the hyponitrite infiltrating solution.

PREPARATION OF SAMPLES FOR MASS SPECTROMETER ANALYSIS: Immediately after the exposure of the infiltrated samples, they were extracted with hot 70 % ethanol by grinding with washed sea sand in a mortar. The ethanol extracts of the infiltrated leaf samples were then transferred to a modified semimicro Kjeldahl distillation apparatus described by Varner et al (27) for the separation and analysis of the soluble nitrogen components of plant extracts. The soluble nitrogen compounds of the ethanol extracts were separated into ammonia plus amide, nitrate plus nitrite and amino acid plus other soluble nitrogen fractions. The resulting nitrogen fractions, in the form of ammonium sulfate, were then oxidized to nitrogen with alkaline sodium hypobromite by the procedures of Rittenberg (23). The nitrogen of each sample was

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[2] Parts of this paper are taken from the dissertation presented by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy at The Ohio State University, 1955.

[3] Present address: Department of Agricultural Chemistry, North Dakota Agricultural College, Fargo, North Dakota.

then analyzed in the mass spectrometer for atom percent excess N^{15}.

Results

Analysis of N^{15}-Labeled Sodium Hyponitrite Preparations: Labeled sodium hyponitrite preparations were found to be of high purity but low in yield. Yields of labeled sodium hyponitrite were approximately 10 % and purity was 90 to 98 %. According to Divers (6, 8) sodium nitrate, sodium nitrite, hydroxylamine, sodium carbonate and sodium hydroxide are generally occurring impurities in the preparation of hyponitrite by the reduction of nitrate with sodium amalgam. Since other N^{15}-labeled nitrogen compounds present as impurities would give erroneous results in any infiltration experiments, it was necessary to analyze the N^{15}-labeled sodium hyponitrite preparations for nitrate, nitrite, hydroxylamine and ammonia. Nitrate and nitrite impurities were found to be less than 0.1 % in each case. No hydroxylamine or ammonia could be detected in the labeled sodium hyponitrite preparations. The remaining impurities were assumed to be non-nitrogenous sodium carbonate and sodium hydroxide. The results of the analysis of a typical N^{15}-labeled sodium hyponitrite preparation are shown in table I. This labeled sodium hyponitrite preparation was used in the infiltration experiment reported in this paper.

Stability of Sodium Hyponitrite: The stability of aqueous solutions of sodium hyponitrite to be used in one hour in vivo experiments and the nature of the decomposition products of sodium hyponitrite under the experimental conditions to be used had to be determined before interpreting the results of any infiltration experiments.

Aqueous solutions of sodium hyponitrite, the salt of a weak acid and a strong base, are slightly alkaline. Various investigators (2, 3, 4, 10, 18, 20, 21, 22) have reported that sodium hyponitrite is quite stable in aqueous solutions at pH 7 or above. Under acidic conditions or on boiling, however, aqueous solutions of sodium hyponitrite have been found to be very unstable and decompose rapidly into sodium hydroxide and nitrous oxide (1, 3, 17, 18, 20). Partington and Shah (22) have reported that sodium hyponitrite decomposes to form nitrogen and nitrite. Chaudhary, Wilson and Roberts (4) have recently demonstrated, however, that no significant amounts of nitrite are formed in the decomposition of aqueous solutions of hyponitrous acid during a 48-hour period.

Table I

Results of the Analysis of a Labeled Sodium Hyponitrite Preparation

Theoretical yield	2.34 g
Actual yield	0.21 g
Percent yield	9.00 %
Percent purity	90.00 %
* Impurities:	
Sodium nitrate	0.08 %
Sodium nitrite	0.05 %
Hydroxylamine	0.00 %
Ammonia	0.00 %

* Other impurities not shown were assumed to be principally sodium carbonate and sodium hydroxide.

Table II

Stability of Sodium Hyponitrite in Aqueous Solution at pH 11.3

Time in minutes	Micromoles of sodium hyponitrite	Micromoles of nitrite
0	56.0	....
5	55.8	0.075
15	55.7	0.070
30	55.6	0.085
60	55.5	0.075
120	...	0.080

Table II illustrates the results of a typical experiment on the stability of sodium hyponitrite in aqueous solution at pH 11.3. A 0.0301-g sample of sodium hyponitrite (98.6 % pure) was dissolved in 50.0 ml of distilled water and aliquots were taken at specified time intervals for the analysis of sodium hyponitrite and nitrite by the procedures mentioned earlier. The results clearly demonstrate the stability of an aqueous solution of sodium hyponitrite and show that nitrite, other than that which was originally present as an impurity of the sodium hyponitrite, is not a decomposition product of sodium hyponitrite under the conditions of the experiment. Sodium hyponitrite solutions buffered to pH 7.3 with 0.3 M Tris (hydroxymethyl) aminomethane also decompose to produce nitrous oxide as the only nitrogen containing decomposition product. The rates of decomposition of sodium hyponitrite solutions at lower pH values are, however, greatly increased. At pH 7.3 a 0.01 M sodium hyponitrite solution has a half life of approximately 11 minutes. This increased rate of decomposition at pH 7.3 agrees rather closely with the decomposition rates of sodium hyponitrite solutions reported by Bonner and Bigeleisen (3) who found that sodium hyponitrite solutions completely decompose in a matter of hours even at a pH of 10.6.

Sodium hyponitrite is not reduced to ammonia by ordinary reducing agents according to Partington and Shah (22). Hyponitrite can be fairly readily oxidized, however, by strong oxidizing agents. Chaudhary, Wilson and Roberts (4) and Medina and Nicholas (12) have reported the oxidation of hyponitrite to nitrite and nitrous oxide with an iodine-acetic acid reagent. The nitrogen of an aqueous solution of sodium hyponitrite was not recovered as ammonia nitrogen using the procedure of Varner et al (27) in the separation and determination of the soluble nitrogen fractions. Therefore, any hyponitrite remaining in the ethanol extracted samples was not oxidized or reduced, but decomposed and lost as nitrous oxide in the alkaline steam distillation of the ammonia-amide nitrogen fraction.

REDUCTION OF INFILTRATED N^{15}-LABELED SODIUM HYPONITRITE BY SOYBEAN LEAVES: Previous experiments (10) have shown that the N^{15}-nitrogen of hyponitrite is reduced to the level of ammonia by infiltrated sections of soybean leaves. In these experiments, however, the nitrate-nitrite nitrogen fractions were too small to be accurately analyzed in the mass spectrometer. Therefore, some doubt existed as to the possibility that some or all of the hyponitrite was first oxidized to nitrite or nitrate before being reduced to ammonia in the infiltrated soybean leaf sections. Table III shows the results of a recent infiltration experiment with N^{15}-labeled sodium hyponitrite in which the N^{15} content of the nitrate-nitrite fractions was sufficient for mass analysis. These results clearly show that the nitrogen of hyponitrite is reduced in the light or the dark to the level of ammonia by infiltrated leaf sections. The highest atom percent excess N^{15}, however, was found to be in the nitrate-nitrite nitrogen fractions of both the light and dark exposed samples. Control samples infiltrated with a previously boiled (decomposed) 0.01 M hyponitrite solution were found to contain very small amounts of N^{15} in the various nitrogen fractions. This N^{15} was presumably derived from the N^{15}-labeled nitrate and nitrite impurities present in the hyponitrite preparation and from any N^{15}-labeled decomposition products which might have been formed during the course of the experiment. In all cases, however, the amount of N^{15} present in the control samples was quite small in comparison with that of the corresponding fraction in the samples infiltrated with unboiled hyponitrite.

TABLE III

FATE OF N^{15}-NITROGEN SUPPLIED AS $Na_2N_2{}^{15}O_2$ TO SOYBEAN LEAVES

SAMPLE	NITROGEN FRACTION	ATOM PERCENT EXCESS N^{15}
$Na_2N_2{}^{15}O_2$ in the light	Ammonia and amide	0.112
	Nitrate and nitrite	0.216
	* Amino acid and other soluble nitrogen	0.049
$Na_2N_2{}^{15}O_2$ in the dark	Ammonia and amide	0.037
	Nitrate and nitrite	0.248
	* Amino acid and other soluoble nitrogen	0.012
Boiled $Na_2N_2{}^{15}O_2$ in the light	Ammonia and amide	0.004
	Nitrate and nitrite	0.000
	* Amino acid and other soluble nitrogen	0.000
Boiled $Na_2N_2{}^{15}O_2$ in the dark	Ammonia and amide	0.006
	Nitrate and nitrite	0.002
	* Amino acid and other soluble nitrogen	0.001

* Soluble nitrogen compounds converted to ammonia by Kjeldahl digestion after removal of ammonia, amide, nitrate and nitrite nitrogen.

DISCUSSION

It had been generally supposed that because of the instability of hyponitrite its decomposition products would interfere with, or make extremely doubtful, the results of any time-consuming experiments designed to establish its assimilation by plants. It was believed, however, that an N^{15} tracer experiment of one hour duration with infiltrated tissues or extracts of plants might prove to be feasible and provide some informative results. The results of the analysis of hyponitrite preparations for nitrogen containing impurities, studies of the stability and the decomposition products of aqueous hyponitrite solutions, and the use of adequate control samples demonstrated the practicality of one hour infiltration experiments.

The assimilation of N^{15} from labeled hyponitrite into the reduced soluble nitrogen fractions of infiltrated soybean leaf sections was found to be approximately four times greater in the light than in the dark. This is not surprising in view of the recent findings of numerous investigators. An increase of nitrate assimilation in the light has been recently reported by Mendel and Visser (15) who found that tomato leaf disks were able to reduce nitrate both in the light and in the dark, but the rate of reduction in the light was about 50 % greater than in the dark. Vanecko and Varner (26) demonstrated that nitrite disappearance also proceeds in both the light and darkness, although the rate in the light was much more rapid than in the dark. Evans and Nason (9) have demonstrated the reduction of nitrate to nitrite in the light with grana and purified nitrate reductase from soybean leaves. Vishniac and Ochoa (28, 29) and Tolmach (25) have shown that illuminated chloroplast preparations reduce pyridine nucleotides. In both light and in the dark, therefore, the nitrate reduction process in green plants is associated with hydrogen transfer from reduced pyridine nucleotides derived from enzymatic dehydrogenations or photochemical reduction. These facts suggest that the increased rate of assimilation of hyponitrite nitrogen in the light is probably the result of a greater availability of reduced pyridine nucleotides in the illuminated sample.

The reduction of N^{15}-labeled nitrate and nitrite to the level of ammonia by plant tissues has been reported by Delwiche (5) and Vanecko and Varner (26) respectively but does not, unfortunately, provide any direct information as to the possible pathways and intermediates involved in their reduction. The highest atom percent N^{15} in these experiments was found in the nitrate-nitrite nitrogen fraction of the ethanol extracts indicating the oxidation of hyponitrite to the level of nitrite or nitrate. Medina and Nicholas (12, 13) in their recent studies of ammonia formation from hyponitrite by crude Neurospora extracts did not report evidence for the formation of nitrite or nitrate from hyponitrite. Since hyponitrite is quite easily oxidized under neutral or slightly acidic conditions (4, 12), nitrite could arise nonenzymatically in the cell sap. In these experiments, hyponitrite could have been reduced directly to am-

monia, possibly through the intermediate formation of hydroxylamine, or oxidized to nitrite and reduced by a process not necessarily involving either hyponitrite or hydroxylamine as intermediates. Even though the reduction of hyponitrite nitrogen to the level of ammonia by green plant tissues has been demonstrated by the use of N^{15}, the role of hyponitrite as an intermediate in nitrate reduction is still an open question.

Summary

1. Labeled sodium hyponitrite was prepared in rather low yield with high purity by the reduction of N^{15}-labeled sodium nitrate with sodium amalgam.

2. Aqueous solutions of sodium hyponitrite at pH 11.3 were found to be stable for two hours at room temperature. Sodium hyponitrite solutions buffered at pH 7.3 were found to decompose at a much faster rate and had half lives of approximately 11 minutes.

3. Labeled hyponitrite nitrogen was assimilated by infiltrated soybean leaf sections in the light and in the dark.

4. Hyponitrite nitrogen was partially or completely oxidized to the level of nitrite or nitrate prior to its reduction to the level of ammonia by infiltrated soybean leaves; hence, the results of these experiments neither establish that hyponitrite per se is assimilated, nor that it is an intermediate in the reduction of nitrate or nitrite to ammonia.

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THE EFFECTS OF LIGHT INTENSITY ON THE GROWTH RATES OF GREEN ALGAE[1,2,3]

CONSTANTINE SOROKIN[4] AND ROBERT W. KRAUSS
DEPARTMENT OF BOTANY, UNIVERSITY OF MARYLAND, COLLEGE PARK, MARYLAND

Among the environmental factors affecting the growth rates of unicellular algae, light is frequently at an improper level. In many laboratory cultures used for physiological research the light intensity is too low to permit logarithmic growth. In nature the intensity is well above saturation and may be high enough to inhibit growth during much of the day. The intensities for saturation and inhibition depend on the suitability of other factors of the environment, e.g., temperature, CO_2 level, and nutrient supply. In attempting to predict the performance of an alga under a given set of conditions it is necessary to know its potential under optimum conditions. Such information is basic to the evaluation of physiological studies and to the design and operation of culture apparatus. This paper describes the growth responses of five green algae to different light intensities under comparable environmental conditions.

MATERIAL AND METHODS

The algae for these studies included: *Chlorella pyrenoidosa* from the collection of Dr. Van Niel, Code No. Z.2.2.1. BAD 12/11; *Chlorella vulgaris* from Dr. Pringsheim's collection, No. 211/8b, listed as the one isolated by R. Emerson and used in his work on iron deficiency and photosynthesis; *Scenedesmus obliquus*, No. WH-50, isolated from fresh waters in Woods Hole by R. W. Krauss in 1950; *Chlamydomonas reinhardti*, plus strain, obtained from Dr. G. M. Smith through Dr. Harold C. Bold; and *Chlorella pyrenoidosa*, high-temperature strain 7-11-05, isolated in Texas in 1951 (10).

The cultures were grown in a medium of the following composition (in grams per liter): KNO_3, 1.25; KH_2PO_4, 1.25; $MgSO_4 \cdot 7\,H_2O$, 1.00; $CaCl_2$, 0.0835; H_3BO_3, 0.1142; $FeSO_4 \cdot 7\,H_2O$, 0.0498; $ZnSO_4 \cdot 7\,H_2O$, 0.0882; $MnCl_2 \cdot 4\,H_2O$, 0.0144; MoO_3, 0.0071; $CuSO_4 \cdot 5\,H_2O$, 0.0157; $Co(NO_3)_2 \cdot 6\,H_2O$, 0.0049; ethylenediaminetetraacetic acid (as a chelating agent), 0.5. The pH of the medium was 6.8. Test tubes with inner dimensions of 16 × 150 mm were used as growth vessels and the 10 ml of culture medium which they contained was inoculated from stock cultures maintained on agar slants. The test tubes were fitted with cotton plugs, through which cotton-plugged bubbling tubes were passed to supply the cultures with a 4 % CO_2-in-air mixture. After inoculation the cultures were placed in a constant temperature water bath made of lucite.

Illumination was provided by two batteries of lamps placed on opposite sides of the bath. Light intensities up to 2,000 ft-c were obtained with two batteries of four, white, 40-watt Champion, fluorescent lamps in each. Intensities above 2,000 ft-c were obtained with General Electric, 300-watt, medium-flood lamps. Reduced intensities were achieved by placing wire screens between the light source and the water bath. Light intensities were measured by a recently calibrated Weston illumination meter with the photocell inside the bath at the level of the culture tubes. It is recognized that the actual light intensity received by any cell in the algal suspension may differ from that measured by the photocell. There are two reasons for this discrepancy. First, cultures receive light from both sides of the bath as well as from reflection and scattering of light from other points around the culture tube. This situation is constant during each of subsequent experiments as long as the technique remains unchanged. Nevertheless, data obtained in different laboratories with different sources, different geometry of illumination, and different culture vessels may not be directly comparable. Second, as the culture grows, mutual shading of the cells reduces the actual amount of light received by a particular cell. It has been assumed that this factor does not influence growth as long as measurements indicate that the culture is in the logarithmic phase. Frequent transfers to fresh medium are required to prevent a reduction in growth rate. Deviations of curves from the exponential may also be due to transition phenomena taking place after transfer of cells from agar slants to liquid media. Therefore more than one transfer to fresh liquid media and a

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score of cell generations were often required before the lag phase was passed and growth measurements on steady-state suspensions could be started.

To measure growth, the optical density of the algal suspension was determined periodically by a photoelectric colorimeter with a filter transmitting at 600 mμ. In a given algal suspension optical density is proportional to the density of the algal population in terms of cell numbers or cell protoplasm per volume of the suspension. In using optical density as a measure of growth the assumption is made that as long as the culture is in the exponential phase of growth under steady state conditions the proportionality between optical density and the density of algal population remains constant.

Growth rate, k, is calculated from the integrated growth equation:

$$k = \log_2 \frac{O.D._1}{O.D._0} \times \frac{1}{t},$$

where $O.D._0$ and $O.D._1$ are optical densities at the beginning and end of the time interval, t. By using logarithms to the base 2, and the unit of time as one day, the growth constant, k, becomes equivalent to the number of doublings per day. At least five readings were taken during exponential growth in each experiment at a particular light intensity. Reproducibility of the rate for at least two subsequent experiments was required. The logarithmic growth rate for each light intensity was calculated as an average from two experiments, each experiment conducted in duplicate.

Results

Figures 1 and 2 give the growth rates of five algae plotted against light intensities up to 10,000 ft-c.

Each of the light intensity curves consists of two portions. Illumination up to 2,000 ft-c was provided by fluorescent lamps. Intensities above 2,000 ft-c were from incandescent lamps. The shapes of the curves might not be identical were there only one source of light throughout the intensity range. However, it is probable that the curves would not differ much. Myers (5, 6), using incandescent lamps, obtained a growth curve for a low-temperature strain of *Chlorella pyrenoidosa* similar to the curve reported here for *C. pyrenoidosa* using fluorescent lamps. The two portions of the curve fit rather well and provide a satisfactory continuation for each other.

A typical light intensity curve for growth resolves itself into three distinct portions: 1) a light dependent portion in which the growth rate rises with the increase in light intensity, 2) a light independent portion or plateau, and 3) a light dependent portion in which the rate declines with increase in light intensity. In all these portions the characteristics of the curve depend heavily on the genetic make up of the organism (fig 1), and on external conditions other than the change in light intensity. Of the external conditions temperature is of primary importance (fig 2).

The light dependent portion of the curve may differ in its slope, in the degree of deviation from a straight line, and in the position of the point where it reaches light saturation. The differences in the slope indicate that the efficiency of utilization of incident light energy depends on the genetic make up of the organism and on external conditions.

The differences in the slope of the light intensity curves for growth, as one moves from lower to higher light intensity, were described by Bristol Roach (1) and observed by Phillips (8). They are also clearly indicated by Myers' data (5) though no reference to them was made. A similar phenomenon has also been observed for photosynthesis by Kok (2, 3, 4). The universal occurrence of this phenomenon is better demonstrated by plotting the lower portions of the curves on a larger scale (fig 3). It is more probable that the breaks, as they appear on the curves in figure 3, are due to discontinuity of measurements; the actual changes in the slopes of the curves are probably continuous and gradual.

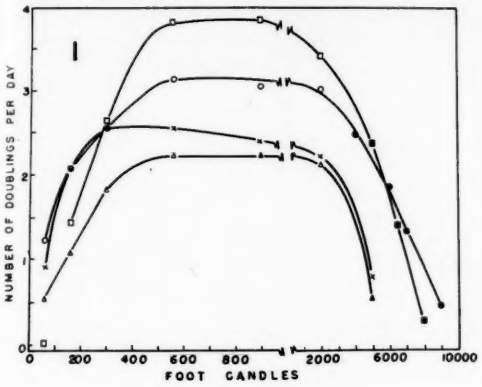
The steepness of the slope of a particular portion of the curve can be calculated in terms of the change in growth rate per 100 ft-c of change in light intensity. These data (a) for the lowest portion of each curve are compared in table I with those for the slopes of the same curves for the interval between 160 and 260 ft-c. Figures in the last column of table I indicate that the growth rates for the 2nd interval constituted only 19 to 58 % of those for the 1st interval. The lowest portions of the light intensity curves must actually have slopes steeper than those shown in figure 3 and the percentage values given in the last column in table I should be even lower. A zero point for growth cannot lie at the zero light intensity but should be moved up to a light intensity at which

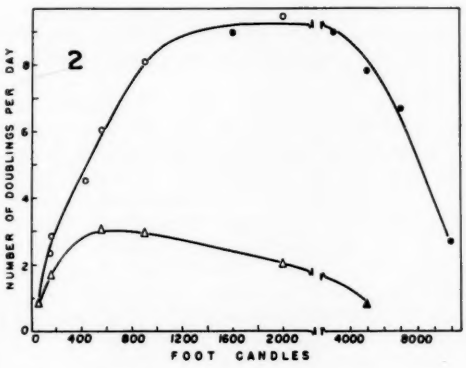
Table I

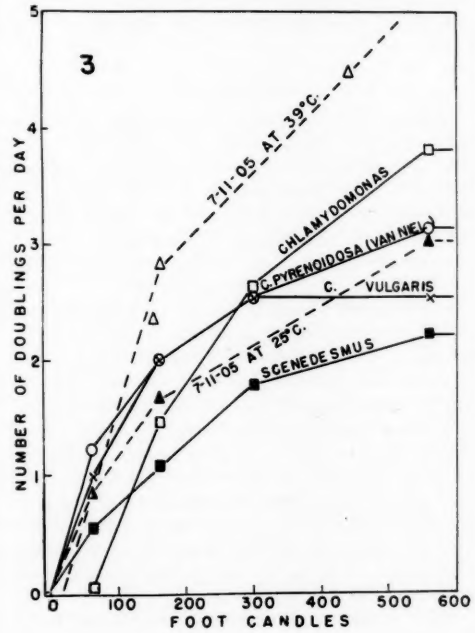
Increase in the Growth Rate (a) in Units of Log_2 per 100 ft-c for Six Green Algae

Organism	Temperature, °C	a units for the intervals of the curves: 0 to 100 ft-c	160 to 260 ft-c: Absolute	160 to 260 ft-c: Per cent
Chlorella pyrenoidosa, (van Niel)	25	2.00	.38	19
Chlorella pyrenoidosa, Emerson (from Myers, 5) *	25	1.20	.56	47
Chlorella vulgaris	25	1.64	.39	24
Scenedesmus obliquus, WH 50	25	.88	.50	57
Chlamydomonas reinhardti	25	1.43	.83	58
Chlorella pyrenoidosa, 7-11-05	25	1.36	.35	26
Chlorella pyrenoidosa, 7-11-05	39	1.70	.60	35

* Recalculated for a saturating light intensity of 400 ft-c according to the correction of Myers (6).







losses by respiration are just compensated by formative gains. For Chlamydomonas this compensation point for growth is above 60 ft-c; for other strains it lies at lower light intensities.

It can be concluded from table I that considered in terms of growth the maximum efficiency of utilization of incident energy, observed at the lowest light intensity, differed widely with various strains. It was more than twice as high in *C. pyrenoidosa* (van Niel) than in Scenedesmus. In *C. pyrenoidosa*, strain 7-11-05, it was higher at 39 ° than at 25° C.

A relatively high efficiency of utilization of incident energy at low light intensities is of great ecological importance. Because of this, an alga may be able to survive better in shaded or deep water. Efficiency must also be considered in predicting yields of mass cultures, since the density of algal populations often limits the light intensity received by the algal cells. It is obvious that any discussion on the utilization of light energy may be of little value unless the dependence of the " efficiency " of the species or strain on the environmental factors employed is clearly recognized.

Because of the gradual transition from the light-dependent to light-independent portion of an intensity curve, the lowest intensity which gives light saturation can only be approximated. For *Chlorella vulgaris* the data obtained in these experiments (table II) indicate that an increase in light intensity above 250 to 300 ft-c did not result in any increase in growth rate. For other strains at 25° C, the value was about 500 ft-c. This value is close to that given by Myers (6) and by Phillips and Myers (9) for the light-saturating intensity for the Emerson strain of *Chlorella pyrenoidosa*. Myers (6) estimated it to be in the neighborhood of 400 ft-c and Phillips and Myers (9), using different technique, found it at approximately 600 ft-c. It should be recognized, of course, that such determinations depend on the light source and the geometry of illumination. A characteristic feature of *Chlorella pyrenoidosa*, strain 7-11-05, is its higher requirement for light saturation at 39° C than at 25° C. Figure 2 shows that higher temperature will be of ad-

FIG. 1. The growth rates of four species of algae at 25° C measured at limiting, saturating, and inhibiting light intensities. The symbols are as follows: *Chlorella pyrenoidosa* (van Niel), circles; *Chlorella vulgaris*, crosses; *Scenedesmus obliquus*, triangles; and *Chlamydomonas reinhardti*, squares. Open symbols show growth under fluorescent light; closed symbols show growth under incandescent light.

FIG. 2. The growth rates of *Chlorella pyrenoidosa*, strain 7-11-05, measured at limiting, saturating, and inhibiting light intensities. Circles plot rates at 39° C; triangles plot rates at 25° C. Open symbols show growth under fluorescent light; closed symbols show growth under incandescent light.

FIG. 3. The growth rates of five strains of green algae measured at limiting light intensities. Data are those from figures 1 and 2 replotted on a larger scale.

TABLE II

GROWTH CHARACTERISTICS OF FIVE STRAINS OF GREEN ALGAE

ORGANISM	TEMPERATURE, ° C	SATURATION LIGHT INTENSITY, FT-C	GROWTH RATE AT HALF SATURATION	GROWTH RATE AT LIGHT SATURATION	LIGHT INTENSITY AT WHICH THE GROWTH RATE DECLINES TO ONE HALF OF ITS LIGHT-SATURATION VALUE
(1)	(2)	(3)	(4)	(5)	(6)
Chlorella pyrenoidosa (van Niel)	25	500	2.4	3.1	6500
Chlorella vulgaris	25	250	1.9	2.6	4500
Scenedesmus obliquus, WH 50	25	500	1.5	2.2	4500
Chlamydomonas reinhardti	25	500	2.2	3.8	6000
Chlorella pyrenoidosa, 7-11-05	25	500	2.3	3.0	3500
Chlorella pyrenoidosa, 7-11-05	39	1400	7.0	9.2	8500

vantage to this strain at any light intensity. An overall comparison of strains in the light-dependent portions of the light intensity curves is provided by the growth rates at half saturation (column 4 in table II). For *C. pyrenoidosa* 7-11-05 at 25° C the half saturating growth rate was practically the same as for *C. pyrenoidosa* (van Niel). At 39° C the growth rate for 7-11-05 was three times higher than at 25° C. The lowest rate at half saturating intensity was for Scenedesmus.

The maximum growth rate exhibited by cells at light saturation differed widely with the strain (column 5 in table II). At 25° C it was almost twice as high for Chlamydomonas as for Scenedesmus. The effect of temperature was more pronounced. For the *Chlorella pyrenoidosa*, strain 7-11-05 at 39° C the growth rate was more than three times higher than at 25° C.

The injurious effects of high light intensities upon algal growth have long been known but no detailed description of the phenomenon is available. As seen from figure 1 the deleterious effects of light at 25° C began at an unexpectedly low light intensity. The light-independent plateaus for the van Niel strain of *C. pyrenoidosa* and Scenedesmus extended from 500 to about 2,000 ft-c. It was shorter for Chlamydomonas, stretching from 500 to not more than 1,000 ft-c. A short plateau from 250 or 300 to not more than 600 ft-c is characteristic of *C. vulgaris*. The low light-saturating intensity and the low intensity injurious to growth of this strain are features characteristic of shade plants.

The description of an organism as a shade plant can be only relative. *C. pyrenoidosa* strain 7-11-05 at 25° C is very sensitive to increasing light intensities (fig 2). Injurious effects began at about 1,000 ft-c. At 39° C this strain showed no deleterious effects of high light intensity up to and probably above 3,000 ft-c. Because of the uncertainty as to the point where the light-independent portion (plateau) of the light intensity curve starts to bend, the light intensity at which the growth rate is depressed to one half of its maximum value is given in column 6 of the table II. The differences between the strains and the importance of the temperature factor are clearly evident.

Working with another strain of *Chlorella pyrenoidosa* Phillips and Myers (9) did not detect injurious effects of light intensities on growth up to 6,000 ft-c. However, differences in technique may provide an explanation for the discrepancy between their data and ours. Their experiments were performed in a flat growth chamber 1.0 cm thick with unilateral illumination. The density of algal populations was kept at a constant and much higher level than the initial optical density in the experiments reported here. Consequently mutual shading of cells may also have played a part in the absence of inhibition at their highest intensity. In the present study the mutual shading of cells was reduced to a minimum.

SUMMARY

The growth rates of *Chlorella pyrenoidosa* (van Niel), *Chlorella pyrenoidosa*, 7-11-05, *Chlorella vulgaris*, *Scenedesmus obliquus*, and *Chlamydomonas reinhardti* were determined under light intensities up to 10,000 ft-c. The intensities at which saturation was developed were shown to vary from 250 ft-c for *Chlorella vulgaris* to 500 ft-c for the other species at 25° C. At 39° C *Chlorella pyrenoidosa*, 7-11-05, was light dependent at intensities below 1,400 ft-c. Inhibition from high light intensities at 25° C began at 600 ft-c for *Chlorella vulgaris* but not until 2,000 ft-c for *Chlorella pyrenoidosa* (van Niel). The other strains were intermediate. *Chlorella pyrenoidosa* 7-11-05 was inhibited above 1,000 ft-c when grown at 25° C but not below 3,000 ft-c at 39° C. The validity of comparisons of these data with those of earlier workers was discussed, particularly with regard to differences in source and geometry of illumination. The growth rates under increasing light intensities were shown to be a function of the genetic make up of the species and temperature.

The change in slopes of the growth rate curves in the light dependent ranges were described for each

species. It appears that a decrease in the slope of the light intensity curve is a characteristic common to these algae and others that have been studied previously.

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THE OXYGEN AFFINITY OF A FLAVIN OXIDASE INVOLVED IN THE RESPIRATION OF STREPTOCOCCUS FAECALIS[1,2]

DONALD J. NIEDERPRUEM and DAVID P. HACKETT

Department of Biology, The University of Buffalo, Buffalo 14, New York

The characterization of the oxidases involved in cell respiration has been based largely on experiments with enzyme inhibitors. In addition, it is possible to characterize an oxidase by its affinity for oxygen, which may be determined by measuring the oxygen consumption as a function of oxygen concentration. This information is especially valuable in those cases where the tissue respiration is insensitive to such classical inhibitors as cyanide and carbon monoxide (see 1, 4, 6). Although it has often been assumed that this "insensitive" respiration is mediated by oxidases which are flavoproteins, recent studies (15, 17) have shown that these same tissues may exhibit a high oxygen affinity, much greater than that exhibited by most isolated flavin oxidases (12). This poses the following question: are there some other flavin oxidases with a relatively high oxygen affinity which could account for this type of respiration? To answer this, a study was made with *Streptococcus faecalis* (B33A) whose respiration is known to be mediated by a flavoprotein oxidase (2, 3). The great advantage of this particular organism is that it does not contain any cytochromes (13), which might complicate the interpretation of the results.

The culture methods employed were those described previously by Seeley and Vandemark (11). The cells were harvested in a centrifuge, resuspended in distilled water, and their respiratory rates determined, in the presence of glucose, by standard manometric techniques at 37.5° C (16). To test the effect of cyanide on respiration Robbie's $Ca(CN)_2$—$Ca(OH)_2$ mixtures were added to the center well (10). Cell-free extracts were obtained by grinding freshly harvested cells with alumina (A-301, Alcoa) in phosphate buffer (0.05 M, pH 7). The slurry was centrifuged at 2,000 × G for 30 minutes in the cold and the supernatant fraction, referred to as the enzyme solution, was used in the spectrophotometric assays. The oxidation of reduced diphosphopyridine nucleotide (DPNH) was followed at 340 $m\mu$ in a Beckmann DU spectrophotometer at room temperature, using cuvettes with a 1 cm light path. The effects of oxygen concentration were determined in vaccine-stoppered cuvettes which had been flushed 10 minutes with the appropriate gas mixtures. To obtain complete anaerobiosis, the special cell of Lazarow and Cooperstein was employed (8). Optical density units were converted to DPNH concentration by assuming a molecular extinction coefficient of 6.22×10^6 sq cm/mole (5). Protein was determined by the method of Stadtman et al (14).

In preliminary experiments, the well known fact that the respiration of *S. faecalis* is not inhibited by cyanide (9, 11) was confirmed using 0.001 M HCN. Following this, the effect of oxygen concentration on the oxygen consumption of whole cells was determined manometrically. The respiratory rates in vessels gassed with 5, 20 and 100 % O_2 showed only slight differences, whether the vessels were gassed initially or after a period in air. The rate in 5 % O_2 was not

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significantly lower than that in air, but there was a slight inhibition, averaging 18 % in three experiments, when the gas phase was 100 % O_2.

These results suggested that the enzyme which mediates respiration here is not a typical terminal oxidase of the flavoprotein type; its properties were therefore examined in cell-free systems. Dolin (2, 3) has shown that extracts of vacuum-dried *S. faecalis* cells contain an active DPNH oxidase system involved in the following two reactions, both of which are catalyzed by flavoproteins:

$$DPNH + H^+ + O_2 \longrightarrow DPN^+ + H_2O_2$$
$$DPNH + H^+ + H_2O_2 \longrightarrow DPN^+ + 2\ H_2O$$
$$2\ DPNH + 2\ H^+ + O_2 \longrightarrow 2\ DPN^+ + 2\ H_2O$$

In the present study, a similar extract was obtained by grinding freshly harvested cells with alumina. This extract also rapidly oxidizes DPNH (0.1 micromole DPNH/min × mg protein), and the formation of DPN as a reaction product was demonstrated by subsequently regenerating DPNH enzymatically. The reaction does not take place with boiled extracts and the rate of oxidation is directly proportional to the volume of extract employed. The oxidase system is not inhibited by a variety of compounds which bind heavy metals, including cyanide, fluoride, phenylthiourea, ethylenediamine tetraacetate and diethyldithiocarbamate (all at 10^{-3} M). On the other hand, it can be essentially completely blocked by the sulfhydryl inhibitor phenylmercuric acetate (10^{-3} M). In the presence of this inhibitor, the DPNH peroxidase reaction can be demonstrated by adding H_2O_2; this suggests that the essential thiol groups are involved primarily in the oxidase portion of the overall reaction. The assumption that this isolated oxidase is part of the normal respiratory pathway is supported by the fact that the respiration of intact cells is insensitive to metal-binding reagents but blocked by phenylmercuric acetate. Further, the measured rates of DPNH oxidation (equivalent to 70 μl O_2/hr × mg protein) could account for all of the respiration of intact cells in the presence of glucose.

The enzymatic oxidation of DPNH by the extract depends on the presence of molecular oxygen, as shown by the fact that there is no reaction under strictly anaerobic conditions. The initial rate of the reaction is not significantly different in mixtures saturated with 5, 20 and 100 % O_2. In order to evaluate the oxygen affinity more precisely, the amount of oxygen in the reaction mixture was adjusted so as to permit an estimate of the oxygen concentration at which the DPNH oxidase proceeds at one half the maximum rate. By carefully regulating the nitrogen flushing, it was possible to leave only enough oxygen to permit a brief oxidation of DPNH before the system became completely anaerobic (fig 1). From the time course curve it is possible to estimate the point at which the rate of DPNH oxidation is one half that in the air-saturated control. From the difference in extinction between the point at which DPNH oxidation stops completely and the point at which the rate

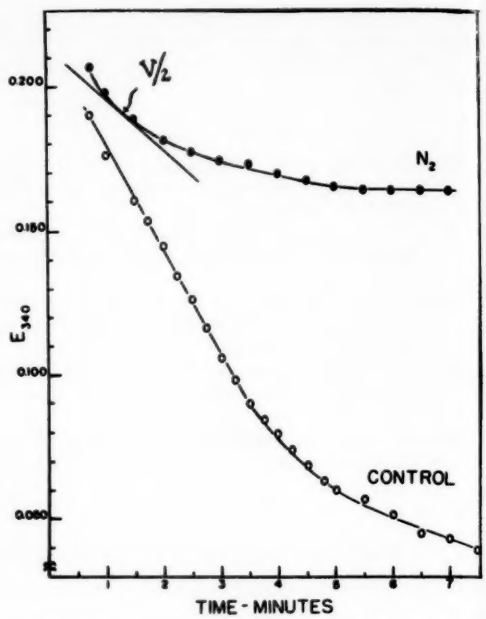

FIG. 1. The rate of DPNH oxidation by *S. faecalis* extracts in mixtures saturated with air (Control) or sufficient nitrogen to permit only a brief oxidation before becoming anaerobic (N_2). The half-maximal velocity (V/2) determined from the latter reaction was used to calculate the oxygen affinity of the oxidase (see text). Reaction mixtures contained: potassium phosphate buffer, 0.05 M, pH 7.0, 1.5 ml; DPNH, 1.8×10^{-4} M, 0.5 ml; extract, 0.05 ml; water to 3.0 ml.

is one half maximal, the corresponding amount of DPNH oxidized can be calculated. In a typical experiment, this value was 4.18×10^{-6} M. Since the overall stoichiometry of the reaction indicates that the DPNH oxidation requires one half the molar concentration of O_2, it follows that the O_2 concentration when the rate of DPNH oxidation was half-maximal ($[O_2]^{50}$) was 2.09×10^{-6} M. Accordingly, the oxygen affinity ($1/[O_2]^{50}$ or $1/K_m$) of the cell-free DPNH oxidase system is 4.8×10^5 M.

Most isolated flavin oxidases have a relatively low oxygen affinity; their activities are generally two to three times greater in 100 % O_2 than in air and markedly reduced in 5 % O_2 (12). Neither the respiratory rate of intact *S. faecalis* cells nor the activity of the extracted DPNH oxidase exhibits this type of dependence on oxygen tension. The actual values of the oxygen affinities of two isolated flavin oxidases are 1.4×10^4 M for xanthine oxidase and 2×10^4 M for amino acid oxidase (7). The affinity of 4.8×10^5 M calculated here for the DPNH oxidation system is more than 10 times greater than these values. Since the reaction with oxygen in this system is catalyzed by a flavin oxidase (2, 3), we must conclude that

there may be flavin oxidases with relatively high oxygen affinities which can be involved in normal respiration.

It has been shown with potato tuber slices (15) and an Aroid spadix (17) that higher plant tissues having a cyanide and CO insensitive respiration can have a relatively high oxygen affinity, with a $1/K_m$ equal to 3×10^5 M. Although this value is higher than the affinity of many flavin oxidases, the results of the present study suggest that this group of enzymes cannot be ruled out as terminal oxidases for respiration on this basis alone. However, the evidence that these plant tissues contain a complete cytochrome system which participates in the normal respiration and which may be involved in cyanide-resistant respiration (17) must also be considered before a final conclusion is reached.

Summary

The respiratory rate of *S. faecalis* cells and the activity of the extracted DPNH oxidase are essentially independent of the oxygen tension when the reaction systems are gassed with 5, 20 and 100 % O_2. The oxygen affinity ($1/K_m$) of 4×10^5 M is unusually high for a flavin oxidase, and the implications of this finding for the analysis of respiratory mechanisms are discussed.

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THE RELATIONSHIP OF THE KINETIN AND RED-LIGHT PROMOTIONS OF LETTUCE SEED GERMINATION[1]

CARLOS O. MILLER

Department of Botany, Indiana University, Bloomington, Indiana

Certain plant growth effects produced by application of kinetin strikingly resemble effects resulting from exposure to red light (2, 3, 4, 5). For example, both kinetin and red light promote the expansion of etiolated bean leaf disks and the germination of lettuce seeds. On the basis of such observations, suggestions have been made that there is a close relationship between biochemical activities of kinetin (6-furfurylamino-purine) and reactions controlled by red light. The experiments reported herein have been performed with the hope of learning the nature of this close relationship if it actually exists.

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In the works cited above, the plant material was at some time exposed to at least a small amount of light. Although there was no evidence that such light affected the controls, the possibility that weak light has a very strong influence in the presence of kinetin was not eliminated. Parts of our own experiments were performed under a dim, green light which did not noticeably influence the germination of lettuce seeds wetted with water; the assumption was made, therefore, that it represented a control condition essentially equivalent to darkness. We now know that this is not so; weak light has very large effects on germination provided kinetin has been supplied to the

seeds. Furthermore, the strong promotion of germination by kinetin previously reported (3) must have been considerably dependent upon exposure to small quantities of light. This relationship, of course, does not necessarily apply to results obtained by other investigators with other plant materials. The conclusion concerning lettuce seed germination is supported by the data represented in figure 1. In this and all other experiments reported in this paper, Grand Rapids lettuce seeds (harvested in 1955) were sprinkled into Petri dishes onto pads consisting of three sheets of Whatman no. 1 filter paper wetted with 5 ml of either water or 5×10^{-5} M kinetin. These dishes were kept in absolute darkness at $25 \pm 1°$ C for 16 hours, next exposed to red light for the indicated times, and then returned to darkness. The amount of germination was determined 48 hours later, a seed being regarded as germinated if any part of the embryo protruded through the seed coats. This criterion of germination was selected because kinetin, at the concentration used, inhibits radicle elongation. At lower concentrations, the inhibition is not very marked but the percentage of germination is smaller. Nevertheless, experiments in which only 5×10^{-6} M kinetin was used revealed the same relationships between the kinetin and light effects as observed with higher concentrations; in these experiments, the radicles emerged before any other parts of the embryos. The red light was obtained by filtering radiation from a 15-watt standard daylight fluorescent tube through two sheets of DuPont red cellophane. The exposures were made at a distance of 205 cm from the surface of the tube and, as measured by a thermopile (black body), the amount of energy at the point of exposure was 98 ergs/sq cm × sec. Although kinetin did promote germination to some extent in absolute darkness, it was much more effective if the seeds were briefly exposed to the weak light.

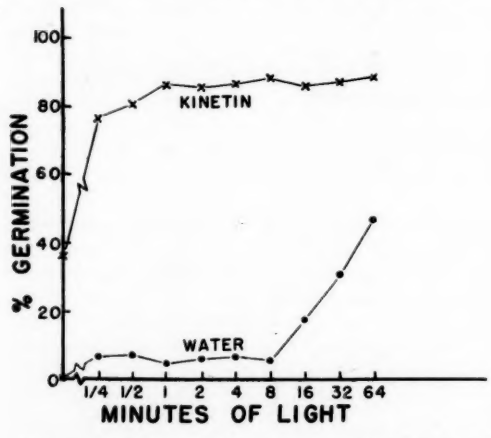

FIG. 1. Promotion of lettuce seed germination by red light in the presence or absence of kinetin. Light exposures at an intensity of 98 ergs/sq cm × sec and for the indicated times. 5×10^{-5} M kinetin was used.

TABLE I

INFLUENCE ON LETTUCE SEED GERMINATION OF COMBINED TREATMENTS WITH KINETIN, RED LIGHT, AND FAR-RED LIGHT

LIGHT TREATMENT	% GERMINATION *	
	ON WATER	ON KINETIN
Darkness	1	34
Red	42	89
Far-red	1	65
Red then far-red	3	64

* Approximately 120 seeds/treatment. 5×10^{-5} M kinetin used.

Only a small percentage of the seeds imbibed on filter paper containing only water responded to the short periods of illumination; with the same exposures, most of the kinetin-soaked seeds germinated. A permissible interpretation of the curves is that kinetin increases the efficiency with which the photoreaction brings about germination.

Further evidence supporting this interpretation has been obtained in attempts to inhibit germination by exposure to far-red light. In our previous report, we stated that far-red does not reverse the kinetin promotion. In the present studies, using a filter system more refined than the one previously employed, we have learned that far-red reverses — but only partially — the promotion caused by exposing kinetin-soaked seeds to red light; and, furthermore, far-red actually promotes germination of kinetin-soaked seeds which otherwise have been kept in the dark! Thus, in a typical experiment (table I), germination of kinetin-soaked seeds illuminated with a high-intensity red light for one minute was 89 % as compared to 34 % for similarly soaked seeds kept in the dark. Exposure to far-red for 15 minutes starting one minute after the end of the red-light exposures decreased the 1st value to 64 % but increased the 2nd value to the same level. The far-red light source used here consisted of one 200-watt tungsten lamp and a filter of two layers of DuPont red cellophane combined with a layer 1 cm thick of a 0.2 % solution of Poirrier's blue. Exposures were made at a distance of 45 cm from the loose end of the tungsten bulb. Energy values were not obtained for this setup, but it was established that exposures of 0.5 minute gave the same results as obtained with exposures of 15 minutes for either promotion or inhibition. Thus, results presented here represent saturation exposures. With this filter system, no transmission was detected in a Beckman DU spectrophotometer in the range 450 to 730 mμ, was less than 0.2 % in the range 400 to 440 mμ and was 4 % at 750 mμ. A possible interpretation of these observations is based upon the fact that the action spectra for promotion and for inhibition of germination overlap and the likelihood that in the region of overlap the two pigments are in equilibrium (1). It may be that some molecules of the pigment involved in promotion are activated at

wavelengths of 730 mμ or longer (as already suggested, 1) and, in the presence of kinetin, quite strongly promote germination. Thus, information obtained by exposures to far-red irradiation also can be interpreted to mean that kinetin increases the sensitivity of the seeds to promotion by red light.

Much can be learned about the relationship being considered here by determining just when kinetin must be supplied to the seeds in order to bring about the increase of sensitivity to red light. In the experiments described above, the kinetin solutions were applied to dry seeds at the beginning of the tests; that is, light exposures were made only after the kinetin solution had been imbibed by the seeds. As a method of supplying the kinetin after the photoreaction had occurred, seeds were imbibed with water for 16 hours, exposed to weak red light, and then transferred to appropriate solutions. These seeds showed little response to kinetin. Thinking that this lack of response might be due to failure of kinetin to get into the seeds prompted the following procedure: Seeds were imbibed on water-soaked filter paper in the dark for 16 hours and then some were exposed to bright red light for 8 minutes. Next, the seeds were immediately blotted and dried over calcium chloride in a desiccator kept in darkness at 25 ± 1° C. Three days later, the dried seeds were transferred to Petri dishes containing filter paper pads wetted with either water or 5×10^{-5} M kinetin. After two additional days in darkness, the germination percentages were determined. Results of a typical experiment are presented in table II. It is quite obvious that kinetin has increased the germination response of the seeds even though supplied after the light reaction was completed. This fairly well eliminates the possibilities that the compound promotes germination by participating directly in the photoreaction or by promoting reactions preparatory to the photoreaction.

Therefore, if kinetin participates directly in a chain of reactions of which the light reaction is also a part, it is very likely that the involvement occurs after photoreaction. Our endeavors to examine this possibility by measuring the influence of kinetin on the rate of dark reactions which immediately follow the photoreaction (1) have yielded no clearly interpretable results. For this reason, the experiments are not described here. It is the author's feeling, however, that all data thus far obtained are consistent with the idea that kinetin does not participate directly in the photoreaction nor in the reactions which immediately precede or follow it. Furthermore, since kinetin at its optimal concentration cannot completely substitute for the light reaction, it cannot be the product of the photoreaction. Although it should be emphasized that these conclusions do not eliminate the possibility of the kinetin and red light actions occurring fairly close together in the same metabolic meshwork, it may be wise for the moment to study each effect separately and intensively.

TABLE II

EFFECT ON LETTUCE SEED GERMINATION OF KINETIN SUPPLIED AFTER EXPOSURE TO RED LIGHT

LIGHT TREATMENT BEFORE DRYING	% GERMINATION *	
	ON WATER	ON KINETIN
Dark	3	29
Red light	28	94

* Approximately 120 seeds/treatment. Seeds imbibed on water, given light treatments, dried, and later germinated. 5×10^{-5} M kinetin used.

Most of the work reported herein was done while the author was still a member of the Botany Department at the University of Wisconsin and was supported in part by a research grant to F. Skoog from the American Cancer Society.

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TRANSLOCATION OF PHOTOSYNTHETIC PRODUCTS TO SOYBEAN NODULES AND THEIR ROLE IN NITROGEN FIXATION[1,2]

MICHAEL K. BACH,[3,4] W. E. MAGEE[3,5] AND R. H. BURRIS

DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF AGRICULTURE, UNIVERSITY OF WISCONSIN
MADISON, WISCONSIN

Nodules from leguminous plants will continue to fix N_2 into acid extractable compounds for several hours after they have been excised from the roots. The capacity of nodules to fix N_2 is decreased two- to five-fold by slicing and is abolished by grinding the nodules (2). The decrease in nitrogen fixation which accompanies slicing of the nodules and suspending them in buffer or nutrient solutions has been attributed to the leaching of essential compounds from the slices. Attempts to restore the N_2-fixing capacity of sliced nodules by the addition of nodule extracts, indoleacetic acid, ascorbic acid, cysteine, glutathione, citrate, succinate, Versene or vitamins have been unsuccessful (2, 11). Virtanen et al (20) observed that if pea plants were kept in the dark before their nodules were harvested, the capacity of the excised nodules for N_2 fixation dropped considerably; upon returning the plants to light, the N_2 fixation of their excised nodules increased to a level intermediate between the dark and light conditions. These experiments, and the earlier experiments of Lindstrom et al (10), clearly established the importance of photosynthetic products for N_2 fixation.

Vernon and Aronoff (19) have employed C^{14} to study the products translocated in soybeans; they found that sucrose was translocated most rapidly and carried the greatest concentration of C^{14}. Nelson and Gorham (13, 14) added labeled sugars to soybean plants and followed their movement through the plants; translocation was much more rapid in the dark than in the light. They also supplied $C^{14}O_2$ and found that products of its fixation were distributed throughout the plant within three hours. Sen and Leopold (17) studied the light and dark fixation of $C^{14}O_2$ in Biloxi soybean. They observed a rapid depletion of sugars and a rise in organic acids and amino acids in the leaves in the dark.

In the present work, $C^{14}O_2$ was furnished to photosynthesizing soybean plants, and the photosynthetic products which were translocated to the roots were detected by their radioactivity. This paper reports the results of these experiments and the effect observed on N_2 fixation by nodule slices when rapidly photosynthesized and translocated sugars were added to media in which the slices were bathed.

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[3] National Science Foundation Predoctoral Fellow.

[4] Present address: Research Department, Union Carbide Chemicals Company, South Charleston, West Virginia.

[5] Present address: Research Division, The Upjohn Company, Kalamazoo, Michigan.

MATERIALS AND METHODS

Soybeans (*Glycine max* Merr., var. Lincoln) were planted in the field or greenhouse. The greenhouse-grown plants were watered with Hoagland and Arnon (7) nitrogen-free solution containing trace elements and double the recommended level of potassium. The field grown plants were fertilized at planting time with commercial 0-10-30 fertilizer. All seeds were inoculated with commercial soybean inoculum.

EXPOSURE TO $C^{14}O_2$: Eight- to 10-week-old plants were used. Barium carbonate labeled with C^{14} was placed in gelatin capsules in a glass dish on the soil next to the plants. Just before sealing a bell jar over the plants, 60 % $HClO_4$ was poured into the glass dish; approximately 20 minutes elapsed before the capsules decomposed and the $C^{14}O_2$ was liberated. The exposures to $C^{14}O_2$ were started during the mornings of bright days and continued for several hours; the bell jars were cooled by flowing water over them. After removal of the bell jars, the plants were allowed to photosynthesize normally for the remainder of the day (day plants), and some plants were not harvested until the following morning (night plants).

HARVESTING, EXTRACTION AND FRACTIONATION PROCEDURES: The root and nodule tissues were separated, and then each sample was treated in a Waring blendor to facilitate subsequent extraction with boiling 70 % ethanol. The insoluble matter was removed by centrifugation, and the clear yellow-green extracts were concentrated under vacuum. A second sediment, which separated during concentration, was discarded. A primary fractionation into amino acids, organic acids and neutral compounds was accomplished with ion exchange resins. A 10×80 mm column of Dowex-50(H^+) held the amino acids; these were eluted with 6 *N* HCl. The water effluent from the Dowex-50 column was passed through a 15×30 mm column of Amberlite IR-45 (OH^-); this retained the anionic compounds (largely organic acids and phosphate esters) which later were eluted with 1 *N* NH_4OH. The material that was not held on either column will be referred to as the "neutral fraction."

The organic acids were separated by the method of Lieberman (9) on a 14 to 15 mm inside diameter column containing 8 g of Mallinckrodt's analytical reagent 100-mesh silicic acid carrying 5.4 ml of 0.5 *N* sulfuric acid (aqueous phase from 475 ml chloroform, 25 ml *n*-butanol and 20 ml 0.5 *N* sulfuric acid) as the non-mobile phase. The column was poured as a slurry in 20 ml of the 5 % solvent. The column was developed at room temperature by starting with the 5 % butanol-chloroform solvent described and in-

creasing the polarity of the solvent by adding 40 % tanol-60 % chloroform (150 ml *n*-butanol equilibrated with 300 ml chloroform and 20 ml 0.5 *N* sulfuric acid; the aqueous phase was discarded and 50 ml butanol was added to under-saturate the solvent with the non-mobile phase). A 125-ml suction flask containing 85 ml of the 5 % solvent served as a mixing vessel, and the 40 % solvent was added from a reservoir by air pressure.

The organic acids also were identified by cochromatography with knowns on columns of Dowex-1 in the formate form as described by Busch et al (5). The sugars in the neutral fraction were separated as their borate complexes by the method of Khym and Zill (8) and Zill et al (22). Hexoses and polyhexoses were estimated with an anthrone reagent (12), whereas pentoses were determined with an orcinol reagent (3). Measurements of radioactivity, and colorimetric or titrimetric analyses were performed on each chromatographic fraction before the samples were pooled. The sugars were identified and their purity determined by paper chromatography with a solvent system containing *n*-butanol, pyridine and water (10 : 3 : 3). The sugar spots, other than sucrose, were detected by the method of Sattler and Zerban (16) with a *p*-aminohippuric acid dip reagent and the sucrose with a naphthoresorcinol spray (4). The sugar chromatograms were developed for 48 hours in descending fashion, as the sugars move very slowly in the solvent used. The relative abundance of C^{14} in the various sugars was determined by scanning one directional paper strip chromatograms of the sugar fractions in a recording radioactivity ratemeter and comparing the areas under the radioactivity peaks plotted by the instrument.

WET ASHING AND COUNTING METHODS: The green tissues (stems and leaves), as well as the residues from the alcohol extraction of the roots and nodules, were dried at 100° C in a forced draft oven and ground in a micro Wiley mill. Aliquots of these tissues were oxidized, and the CO_2 was collected as $BaCO_3$ (18). The carbon content of the samples was determined by titration of the excess $Ba(OH)_2$. The $BaCO_3$ was separated by centrifugation, washed and resuspended in methanol, and aliquots of the suspension plated on tared aluminum planchets and counted with a thin-window Geiger counter. Corrections were made for background, self-absorption and coincidence, and specific activities were calculated. The radioactivity of the fractions recovered chromatographically was determined by plating aliquots (the whole fractions from the organic acid columns were used) on cupped aluminum planchets, drying, and counting under a thin-window Geiger tube.

EXPOSURE TO N_2^{15} AND ANALYSIS FOR N^{15} INCORPORATION: Nodules were exposed to N_2^{15} in two types of containers. Small Warburg flasks were used in following the time course of N_2 fixation. The second type of container was a specially constructed Lucite disk (fig 1) with 1-inch holes drilled out to serve as receptacles for 25-mm diameter, 7-mm high stainless steel dishes. A partial vacuum was maintained in the container, and an O-ring formed a seal between the top and bottom parts. Exposures were for two hours at 25° C, under an atmosphere consisting of 10 % N_2^{15} (30 atom percent N^{15} excess), 40 % O_2 and He to 1 or 0.8 atmospheres, depending on whether the first or second type of container was used. The nodules were inactivated and extracted by grinding with 3 *N* HCl, centrifuged, and the soluble portion analyzed for N^{15} (2).

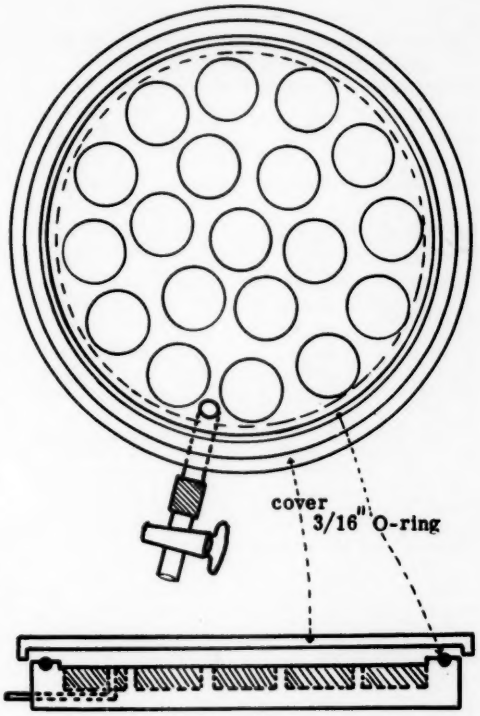

FIG. 1. Lucite chamber for exposing excised nodules to N_2^{15}. The diameter of the cover is 7.5 inches, and the diameter of the dish is 7 inches. The O-ring is held in a groove in the dish. The area inside the dashed circle (top view) is recessed 1/16 inch; the 1 inch diameter, 3/8 inch deep holes are cut into this recessed area. The chamber is evacuated and gassed through the stopcock.

RESULTS AND DISCUSSION

Table I gives the percentage total and relative specific radioactivities of the leaves, stems, roots and nodules of the plants which were exposed to 5 mc $C^{14}O_2$. One half of the plants in this experiment were harvested just after sunset (10 hours after the beginning of the exposure to $C^{14}O_2$) and are referred to as the day plants. The rest of the plants (night plants) were harvested just before sunrise on the following day (19 hours after the beginning of the exposure to $C^{14}O_2$).

TABLE I

GROSS DISTRIBUTION OF C^{14} IN SOYBEAN PLANTS AFTER EXPOSURE TO 5 MC $C^{14}O_2$*

TISSUE	TOTAL CPM × 10^{-6}	% OF TOTAL RECOVERED C^{14}	SPECIFIC RADIOACTIVITY CPM × 10^{-3}/MG DRY WT
Day plants			
Leaves	86.59	55.4	9.41
Stems	54.24	34.3	4.34
Roots	11.74	7.6	3.14
Nodules	3.78	2.9	4.20
Night plants			
Leaves	44.74	62.2	8.18
Stems	21.31	29.5	5.61
Roots	4.02	5.5	1.39
Nodules	1.91	2.7	2.89

* The data for leaves and stems are based on analyses of the dried tissues which were wet ashed, whereas those for the roots and nodules represent the sum of the C^{14} in the residue and extract obtained with boiling 70% ethanol. The day roots contained 85% of their total C^{14} in nonextracted form, the day nodules 23%, the night roots 52%, and the night nodules 18%.

Although the leaves had the highest C^{14} specific activities (table I), the specific activities of the nodules in the day and night samples were respectively 1.34 and 2.08 times those of the roots. This is reasonable, as the nodules are more active metabolically and have less woody tissues than the roots. The specific activity of the leaves decreased somewhat from day to night while that of the stems increased. This could arise from translocation of the C^{14}-labeled material from the leaves. The specific activities of the roots and nodules also decreased during the night, possibly because their C^{14}-labeled compounds were oxidized to $C^{14}O_2$ in preference to their structural, unlabeled carbon compounds.

Investigation of the roots and nodules was directed chiefly at establishing the similarities and differences between the distribution of the C^{14} in these tissues, and particularly the changes in these distributions which occur during a dark period. It was hoped that these comparisons might give some indication concerning the nature of the factors which limit fixation of N_2 in darkened plants relative to plants which are photosynthesizing actively.

Table II gives the distribution of C^{14} among the various fractions from the roots and nodules of the day and night plants as percentages of radioactivity applied to the Dowex-50 and Amberlite IR-45 columns in series. The table reveals interesting differences in the total C^{14} found in organic acids and amino acids. During the day, the organic acids in the roots had nearly twice the percentage of the total C^{14} found in the amino acids, whereas, the amino acids in the nodules had almost twice the percentage C^{14} of the organic acids. After changes had occurred during the night, the percentage C^{14} decreased substantially in the organic acids from the roots but remained relatively constant in the amino acid fractions; in the nodules the organic acids increased in percentage C^{14} about 2.5 times during the night, while the percentage of total C^{14} in the amino acids decreased to less than half of its day value. The proportional changes in the neutral fractions were much smaller, although the absolute changes were comparable.

Virtanen et al (20) reported that fixation of N_2 by the nodules slows down when the plants are kept in the dark. The observation that the percentage of the total C^{14} in free amino acids decreases during the night suggests that the preformed amino acids are incorporated into protein, and little N_2 is fixed in the dark to replenish the supply of free amino acids. Under such conditions one might expect the organic acids to accumulate in the nodules at night, when N_2 fixation is limited, and to be converted to the amino acids only as new N_2 is fixed. Although it would be logical to assume that fixation of N_2 at night might be limited by lack of suitable nitrogen acceptors, it seems clear from the indicated abundance of total organic acids, that their lack cannot be responsible for decreased fixation. It remains possible that a specific organic acid may have been depleted while the total acids increased.

Figure 2 shows a typical separation on a column of silica gel of the organic acids from nodules. The figure records the titration and radioactivity for each fraction. Table III indicates the distribution of C^{14} among the organic acids of the day and night tissues. The compounds were identified by their behavior when cochromatographed with knowns on silica gel and Dowex-1 formate columns. As fractions 3 to 7 were not completely resolved on the silica gel column, no separate values (except for night nodules) are given for them in table III; the calculations for the night nodules were based on further analysis of these samples on Dowex-1 columns. Attempts to identify unknowns X_1 to X_4 failed. X_1 and X_2, although eluted in the region where the volatile organic acids

TABLE II

DISTRIBUTION OF C^{14} AMONG THE GROSS FRACTIONS FROM SOYBEAN ROOTS AND NODULES

FRACTION	ROOTS		NODULES	
	CPM × 10^{-6}	% APPLIED RADIOACTIVITY	CPM × 10^{-6}	% APPLIED RADIOACTIVITY
Day plants				
Neutral	0.783	45.3	1.170	50.2
Organic acids	0.284	16.4	0.182	7.8
Amino acids	0.148	9.3	0.344	14.7
Recovery	1.215	71.0	1.696	72.7
Night plants				
Neutral	0.974	53.3	0.893	56.7
Organic acids	0.219	12.0	0.315	20.1
Amino acids	0.156	8.5	0.103	6.6
Recovery	1.349	73.8	1.311	83.4

TABLE III

DISTRIBUTION OF C^{14} AMONG THE ORGANIC ACIDS FROM SOYBEAN ROOTS AND NODULES AS SEPARATED ON A COLUMN OF SILICA GEL

FRACTION	COMPOUND	% OF TOTAL APPLIED RADIOACTIVITY			
		ROOTS, DAY	ROOTS, NIGHT	NODULES, DAY	NODULES, NIGHT
1	X_1	4.9	0.0	1.7	1.6
2	X_2	1.5	3.4	0.0	0.0
3	Pyruvic	12.0	16.7	4.5	6.7
4	Fumaric				0.6
5	Lactic				0.1
6	X_3				7.0
7	Succinic	3.5		3.1	2.9
8	X_4	3.6	3.1	4.1	5.0
9	Malic	8.3	14.4	15.2	21.8
10	Citric	4.2	2.8	3.4	8.5
Misc.*	...	13.8	10.2	10.8	8.5
Recovery, %		51.8	50.6	42.8	62.7

* C^{14} recovered, but not in specific compounds.

are eluted, are not volatile, as they do not decrease in radioactivity when acidified before plating for C^{14} determination. Compound X_3 accumulated a considerable portion of the total radioactivity of the organic acid fraction. This compound did not cochromatograph with succinate, lactate or glutarate, the only compounds which were mapped by Lieberman (9) in the region of the chromatogram where X_3 appeared. Compound X_4, which is abundant but not very active metabolically, originally was thought to be α-ketoglutarate. However, it failed to form a dinitrophenylhydrazone and did not move with α-ketoglutarate on the Dowex-1 columns; it also did not move with succinate, lactate, cis- or trans-aconitate or glycolate. Nomura et al (15) have described the occurrence in *Monilia formosa* of a compound having similar properties.

Interesting changes appeared in the percentages of the total C^{14} found in the different organic acid fractions of the four samples analyzed. Compound X_1 disappeared from the root samples at night. This suggests that compound X_1 could be a product whose depletion limits fixation. The percentage of total C^{14} in fractions 3 to 7 increased but slightly in the roots, whereas it more than doubled in the nodules from day to night. In contrast, malate increased in both the roots and nodules. Citrate, on the other hand, increased in the nodules and decreased in the roots. The fractionation conditions employed were mild enough to preserve pyruvate, but α-ketoglutarate, a compound of comparable stability, was not found.

Fractionation of the amino acids into acidic amino acids and neutral plus basic amino acids with washed columns of Amberlite IR-4B failed to reveal any changes in the distribution of C^{14} between these fractions from day to night. In all cases the acidic amino acids carried about 30 % of the total C^{14} in the amino acid fractions.

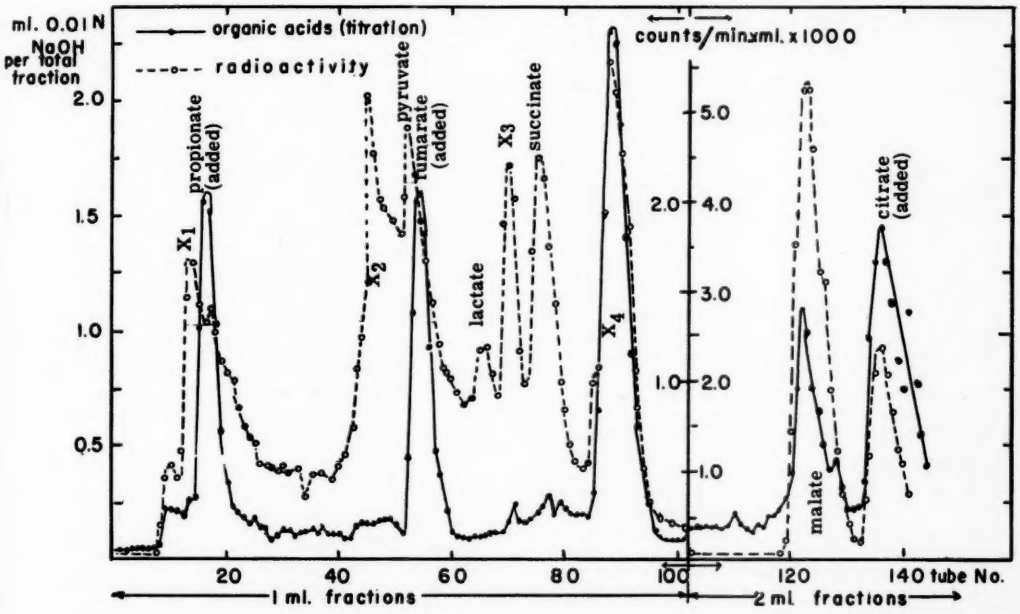

FIG. 2. Separation of the organic acids from C^{14}-labeled soybean nodules on a silica gel column by gradient elution with butanol-chloroform. The plants had been harvested 19 hours (10 hours in the light followed by 9 hours in the dark) after their first exposure to $C^{14}O_2$.

Table IV summarizes the distribution of C^{14} in the neutral fractions from the roots and nodules as based on counts of aliquots from the Dowex-1 columns. When paper chromatograms resolved the fractions from the column into separate compounds, the measurements of radioactivity on the paper chromatograms were used to calculate an appropriate correction for the contribution of the impurities in the Dowex-1 fractions. A number of sugars recovered from the columns of Dowex-1 remain unidentified (table IV). Compound X_6 moved with lactose on paper chromatograms. Mild acid treatment hydrolyzed it to compounds which moved with glucose, xylose and arabinose on chromatograms. All the radioactivity of the compound appeared to be in its hexose. The fact that fructose appeared to increase fixation of N_2 more than the other sugars tested (table VI) suggests that it may be used preferentially in the nodules. This could explain its apparent absence in the day nodules.

Although the results described here were confirmed qualitatively between two similar experiments carried out at different times, there were distinct quantitative differences between the experiments. Probable inaccuracies in the methods employed include incomplete extraction of the tissues, particularly root tissues, and incomplete separation and recovery of sugars on columns of Dowex-1. However, the main cause for variability between experiments probably was the biological variability of the tissues concerned, for the material was taken from the field during two growing seasons. Based on these considerations, the results described here should be taken chiefly as a qualitative indication of the distribution of C^{14} among the various compounds and the interconversions of the compounds in these tissues.

TABLE IV

DISTRIBUTION OF C^{14} AMONG CARBOHYDRATES AND UNKNOWN NEUTRAL COMPOUNDS FROM SOYBEAN ROOTS AND NODULES

COMPOUND OR FRACTION	C^{14} AS % OF TOTAL C^{14} ADDED TO COLUMN			
	ROOTS		NODULES	
	DAY	NIGHT	DAY	NIGHT
X_5*	25.1	...	7.1	5.5
X_6*	13.2	...	10.6	11.6
Sucrose	10.8	12.7	...	...
Glucose **	...	2.4	11.4	5.8
Fructose **	...	1.9	...	4.5
X_7 and X_8*	...	...	9.6	...
Pentose	1.6	0.7	1.1	0.7
Fructose	12.1	4.0	...	5.9
Glucose	10.4	9.7	14.1	1.9
Misc.***	6.2	1.6	3.1	1.5
Recovery, %	79.4	33.0	57.0	37.4

Materials were separated on columns of Dowex-1 borate and by paper chromatography.

Carbohydrate concentrations were determined with anthrone or orcinol.

* X_5 was polymeric material remaining near the origin of paper chromatograms; X_6 apparently was a trisaccharide; X_7 and X_8 were fast moving hexoses released on the paper chromatograms from polymers recovered from the Dowex-1 column.

** These glucose and fructose fractions were released on the paper chromatograms from polymers recovered from the Dowex-1 column, whereas the other glucose and fructose samples were recovered as such from the Dowex-1 column.

*** Material recovered from Dowex-1 borate column but not in specific fractions.

TABLE V

EFFECT OF THE CONCENTRATION OF SUCROSE ON THE INCORPORATION OF N^{15} INTO SLICED SOYBEAN NODULES EXPOSED TO N_2^{15}

MOLAR SUCROSE CONCENTRATION	NO. OF SAMPLES	AVER. ATOM % N^{15} EXCESS	STD. DEV.** OF MEAN	t *** (CALC.)	t *** (FROM TABLE)
0.000	20	0.100	0.010	...	...
0.003	13	0.103	0.012	0.18	0.69 (50)
0.005	7	0.137	0.017	4.10	2.79 (1)
0.010	20	0.135	0.013	2.15	2.02 (5)
0.020	4	0.178	0.035	2.86	2.07 (5)
0.030	3	0.077	0.016	1.31	1.06 (30)
0.050	13	0.089	0.012	0.70	0.69 (50)

* Sliced nodules were exposed to 30 atom % N^{15} excess N_2 for 2 hours at 25° C.

** The standard deviation of the mean of the grouped data is calculated from the equation $d^2 = \frac{\Sigma x^2 - (\Sigma x)^2/n}{[\Sigma(n-1)]n}$.

*** The results were calculated by the t-test (20). The t values from the tables are given with the percentage level of confidence in parentheses for which they are calculated. The comparisons were between samples with and without sucrose.

The demonstration of highly radioactive carbohydrates in the nodules prompted us to investigate their effect upon nitrogen fixation; our earlier work (2, 11) had revealed no compounds which increased fixation. Table V shows how varying the concentrations of sucrose affects the fixation of N_2^{15} by sliced soybean root nodules. Because of the variability in fixation between and within experiments, several separate experiments were necessary to yield results which could be evaluated statistically. The results of each experiment were adjusted so that nodules from the flasks without added sucrose had 0.100 atom percent N^{15} excess. The t-test was applied to these results. Only with 0.005, 0.010 or 0.020 M sucrose was the probability level at 1 % or 5 %; a real increase of fixation by added sucrose is unequivocal in these instances.

Figure 3 depicts the effect of sucrose on the time course of N_2^{15} fixation by sliced soybean root nodules. The control (no added sucrose) samples taken at 120 minutes served as reference samples and were adjusted to 0.100 atom percent N^{15} excess. The vertical lines at each experimental point correspond to the standard deviation of the mean for that point, and the number underneath the point represents the number of individual samples which were used in calculating the mean. Many samples were analyzed, be-

cause the results were quite variable. Although there was only 20 to 40 % confidence that the differences between the fixation of N_2^{15} with and without the addition of sucrose were significant, the results described good curves. Hence, we feel that the enhancing effect of sucrose on N_2 fixation by the nodule slices is real throughout the experiment and is not due to a prolongation of the fixation period beyond that of the controls.

Table VI summarizes the effect of three sugars on the fixation of N_2^{15} by sliced nodules. All sugars were used at 10^{-2} M concentration, and all exposures to N_2^{15} were for two hours at 25° C. A preliminary experiment (suggested by the observations of Gauch and Dugger (6)) had shown that the addition of 5 ppm boron as H_3BO_3 had no enhancing or inhibitory effect when 10^{-2} M sucrose was used as substrate, but boron was added in these experiments on the chance that its effect might be more marked with the other sugars. The samples without added sugar were adjusted to 0.100 atom percent N^{15} excess. The differences among the increases caused by glucose, fructose or sucrose in the experiments of table VI were not statistically significant at the 95 % confidence level.

If the observed decrease in the fixation of N_2 which accompanies slicing or crushing of the nodules is caused by the leaching out of carbohydrates, one would expect that the addition of these compounds to the sliced nodules would restore fixation to the level of the whole nodules. Furthermore, one might expect that the addition of these substances to nodules taken from plants which had been kept in the dark would restore their fixation to a level comparable to that of nodules from plants which had been kept in the light. Neither of these postulates was verified. The maximum increase we observed when carbohydrates were added to sliced nodules was about 60 %, whereas the decrease in fixation which accompanies slicing is usually two- to five-fold. These observations indicate that factors in addition to the removal of the carbohydrates we have tested from sliced nodules must limit their rate of fixation of N_2.

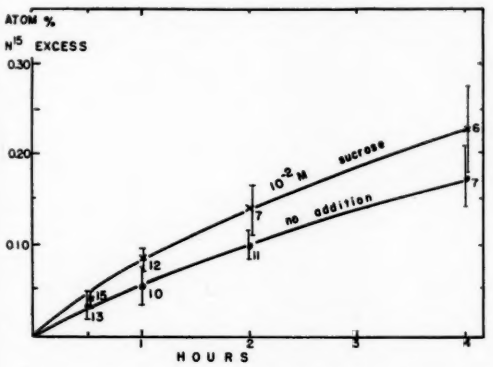

FIG. 3. The effect of added sucrose on the time course of fixation of N_2^{15} by sliced soybean nodules. The sucrose concentration was 0.01 M, and all the slices were floated in 0.05 M phosphate buffer. They were exposed to an atmosphere containing 10 % N_2^{15} (30 atom % N^{15} excess), 40 % O_2 and 50 % He. Results are adjusted to make the sample from the buffer control after a 2 hour exposure equal to 0.100 atom % N^{15} excess. Vertical lines represent standard deviations of the means, and the number adjacent to each point represents the number of determinations.

TABLE VI

EFFECT OF ADDED FRUCTOSE, GLUCOSE OR SUCROSE ON THE FIXATION OF N_2^{15} BY SLICED SOYBEAN NODULES *

TREATMENT	AVER. ATOM % N^{15} EXCESS	NO. OF SAMPLES	STD. DEV. OF MEAN	t *** (CALC.)	t *** (FROM TABLE)
No addition	0.100 **	8	0.010	....	....
0.01 M fructose	0.163	10	0.012	1.35	1.33 (20)
0.01 M glucose	0.127	10	0.008	0.674	0.688 (50)
0.01 M sucrose	0.138	10	0.013	....	....

* All additions were made in 0.05 M phosphate buffer (pH 6.8) containing 5 ppm B as H_3BO_3.

** The concentrations of N^{15} in the samples without added sugars were adjusted to 0.100 atom % N^{15} excess.

*** The t-test was applied to compare the fixation with added sucrose versus the other sugars; the percentage levels of confidence for the t values are given in parentheses.

SUMMARY

The root and nodule tissues of soybean plants, whose tops had been exposed to $C^{14}O_2$, were examined for the distribution of C^{14} among their various organic acids, amino acids and carbohydrates, and for the changes occurring in this distribution when the plants were allowed to metabolize the radioactive material during a dark period following the initial light period. During the day, the organic acids of the roots had a higher percentage of the total C^{14} than did the amino acids; the reverse was true in the nodules. At night, the percentage of total C^{14} in the organic acids of the roots decreased while it increased markedly in the nodules. Several unidentified acids and sugars were found. The addition of sugars to sliced, excised soybean nodules increased their fixation of N_2^{15}. The optimal concentrations of sugar were in the range 0.005 to 0.02 M, and glucose, fructose and sucrose had essentially the same enhancing action. The increase did not arise from a prolongation of the period of N_2 fixation but rather from a continuous enhancement of fixation throughout the exposure to N_2^{15}.

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THE PROPERTIES OF CYTOCHROME OXIDASE IN CHOLATE EXTRACTS FROM SOYBEAN ROOTS[1,2]

GENE W. MILLER, HAROLD J. EVANS and EDWARD SISLER
North Carolina State College, Raleigh, North Carolina

Cytochrome oxidase preparations have been obtained from many different organisms including higher plants (1, 4, 21). Since cytochrome oxidase is firmly bound to the particulate material, fractionation and purification by usual techniques has not been possible. Surface active agents including sodium cholate or sodium deoxycholate have been shown to disperse particulate cytochrome oxidase from heart muscle resulting in a clear extract. Some purification from this material has been obtained (3, 19, 20). Smith (16) reported a 20-fold purification of cytochrome oxidase from a heart muscle extract by use of sodium cholate treatment, trypsin digestion and then fractionation by ammonium sulfate. No information has been found in the published literature concerning the purification of cytochrome oxidase from higher plant sources.

In a previous paper (11) concerning the stimulatory influence of salts on the activity of particulate cytochrome oxidase, it was suggested that electrolytes may be necessary either for the transfer of electrons through the lipoprotein particulates or for the maintenance of the particulate enzyme in the proper orientation to allow access to substrates. If these postulations were correct, one possibly might expect the salt stimulation to disappear if sufficient dispersion of the particulates could be obtained by use of surface active agents. In this paper the properties of cytochrome oxidase are described in two types of extracts prepared from soybean root particulates by

[1] Received October 25, 1957.
[2] Contribution from the Faculty of Botany, North Carolina Agricultural Experiment Station and published with the approval of the Director as paper No. 849.

use of sodium cholate. The experimental results to be presented show that salts failed to stimulate the activity of either of two cholate dispersed fractions. A preliminary report (12) of these findings already has been presented.

Materials and Methods

Particulate Preparation: Roots from soybean seedlings (*Glycine max* Merr.) grown as previously indicated (11) were homogenized for five minutes in two weights (in respect to weight of roots) of cold 0.1 M potassium phosphate buffer at pH 7.4. The homogenization was accomplished by use of an Omni mixer (Ivan Sorvall Inc., Norwalk, Conn.) and the entire preparatory operation was conducted at temperatures between 0 and 4° C. The homogenate was strained through two thicknesses of cheesecloth (fraction I) and the liquid adjusted to pH 5.6 with cold 1 M acetic acid. The precipitate, collected by centrifugation at 25,000 × G for 15 minutes, was suspended in a volume (equal to one fourth the original volume of the homogenate) of 0.1 M phosphate buffer at pH 7.4. A glass Ten Broeck homogenizer was used to suspend the precipitate. This preparation was used as the particulate suspension and is referred to as fraction II.

Preparation of Cholate Extract: A quantity of a 20 % solution of sodium cholate at pH 7.4 was mixed with the particulate suspension to make the final concentration 2 %. The stock solution of sodium cholate in 0.1 M phosphate buffer at pH 7.4 was prepared from cholic acid (purchased from Fisher Scientific Co.) and adjusted to pH 7.4 by use of NaOH. In certain experiments the mixture of sodium cholate and extract was digested with trypsin (18) or with papain, but this treatment had no apparent effect on the fractionation procedure and therefore was not routinely used.

After centrifugation of the cholate extract for 10 minutes at 25,000 × G, the sediment was discarded. The supernatant liquid is referred to as fraction III. Sufficient solid $(NH_4)_2SO_4$ was added to the supernatant to obtain a 30 % saturated solution. The protein from this precipitation was collected by centrifugation and dissolved in a volume of 0.1 M K_2HPO_4 buffer at pH 9.0 equivalent to 20 % of the volume of the supernatant (this is referred to as fraction IV). A protein fraction also was collected between 30 and 65 % saturation with $(NH_4)_2SO_4$ and was dissolved in a volume of 0.1 M K_2HPO_4 buffer at pH 9.0 equivalent to 20 % of the volume of the supernatant (referred to as fraction V [3] in table I). Both of the extracts precipitated by $(NH_4)_2SO_4$ were adjusted with NaOH to a pH of 7.4. Centrifugation for one hour at 25,000 × G of either fraction IV or V resulted in no loss of activity. Centrifugation for one hour at an average force of 66,000 × G decreased the activity of fraction IV to one third that of the original, but an equivalent centrifugation had no effect upon the activity of fraction V. Fraction IV was yellow in color and contained 9.9 % cholate as determined by the method outlined by Reinhold and Wilson (15). The activity of this extract on a protein basis as determined by Folin's phenol reagent (8) was about twice that of the crude extract. Fraction V was colorless and contained 11.0 % cholate. The activity of this extract on a protein basis was about 8 times that of the crude extract. Both extracts were optically clear and retained their activities for several weeks when stored at −15° C. The essential data of the average of three purification experiments are shown in table I. These data are typical of many replications of the purification procedure.

Enzyme Assay Procedures: In the standard cytochrome oxidase assay procedure, enzyme activity was determined spectrophotometrically at 550 mμ with a Beckman DU spectrophotometer (2, 11). The reaction mixture in a volume of 1 ml contained; 85 micromoles of potassium phosphate buffer at pH 7.0, 0.05 micromoles of cytochrome c of 90 % purity (prepared and assayed as previously described (2, 11)) and a quantity of enzyme sufficient to obtain the desired activity. Enzyme activity in this procedure is indicated as the decrease in optical density at 550 mμ during the 15-second interval between 10 and 25 seconds after the enzyme was added to start the reaction. In the purification procedure (table I) it was

[3] In referring to fraction V an enzyme reaction is inferred; however, it is concluded that the nature of the reaction has not been established.

Table I

Purification of Cytochrome Oxidase from Soybean Roots

	Fraction	Volume	Total activity	Recovery	Protein	Specific activity
		ml	*units* *	*%*	*mg/ml*	*units per mg protein*
I	Crude extract	480	399	...	1.8	0.4
II	Acetic acid ppt.	125	199	54.2	1.8	0.8
III	Cholate extract of II	138	262	71.3	1.8	0.95
IV	0 to 30 % $(NH_4)_2SO_4$ ppt. of III	15	30	8.2	2.1	0.75
V	30 to 65 % $(NH_4)_2SO_4$ ppt. of III	18	70	19.0	1.2	3.2

* One unit is defined as a decrease in optical density of 1 per minute during the initial 15 second interval using the assay procedure described in the text.

necessary to compare activities of fractions exhibiting nonlinear rates with those exhibiting linear rates. In these experiments, therefore, the standard procedure was used with the exception that the optical density change for the initial 15-second interval was determined instead of the change during the 10- to 25-second interval. This determination was corrected for the optical density change resulting in dilution by the addition of enzyme.

The data presented in figure 4 were obtained by use of the colorimetric method for determination of cytochrome oxidase (17) which involved the measurement of the rate of oxidation of the leuco form of 2,3',6-trichloroindophenol (obtained from the Eastman Kodak Co.) at 645 mμ. The oxidation of the reduced dye in these experiments was dependent on catalytic concentrations of oxidized cytochrome c.

The addition of crystalline catalase (kindly supplied by Dr. Sam Tove) to the various cytochrome c preparations failed to influence activity and therefore it was concluded that hydrogen peroxide was not present in the cytochrome c solutions.

Other enzymes assayed include DPNH oxidase which was determined spectrophotometrically by the rate of decrease in optical density at 340 mμ (6) and ascorbic acid oxidase activity which was assayed by measurement of the decrease in optical density at 265 mμ (14).

Absorption Spectra: The difference between the spectra of the extracts in the reduced and oxidized forms was obtained by use of a Warren Spectracord. The enzyme was reduced by addition of sodium hydrosulfite to a cuvette of 1 cm light path which contained the enzyme (3). The spectrum in each experiment was recorded within 90 seconds after addition of the reducing agent.

Results

Kinetics of Reactions: The curves in figure 1 illustrate the course of reactions catalyzed by fraction II (acetic acid precipitated particulates), fraction III (cholate extract of particulates) fraction IV (0 to 30 % $(NH_4)_2SO_4$ precipitate (ppt.) from fraction II) and fraction V (30 to 65 % $(NH_4)_2SO_4$ precipitate (ppt.) of fraction III). From these curves it is apparent that the rates of reactions catalyzed by fractions II and IV were linear with time. The reaction rate with the crude extract (fraction I) also was linear; however, this data is not presented. It is generally reported that cytochrome oxidase reactions follow first order kinetics (2, 21) however using the assay system described and the particulate fractions indicated zero-order reactions were consistently obtained. These results are in accord with those of a previous report (11). The rate of the reaction catalyzed by fraction III was diphasic decreasing slightly in the initial phase of the reaction then remaining linear for the remainder of the experimental period. The activity of fraction V fell off rapidly with time and as indicated by the curve (fig 1) most of the reaction took place in the initial 25 seconds. When the logarithm of this decreasing rate was plotted against time the curve failed to indicate a first-order reaction. After 115 seconds the addition of reduced cytochrome c to the reaction mixture containing fraction V failed to accelerate the reaction. The addition of enzyme to a comparable reaction after 100 seconds, however, resulted in a marked decrease in optical density for the ensuing 25-second interval. This suggests that the fraction V preparation presumed to be an enzyme was inactivated by some substance and this point receives further consideration under studies of inhibitors.

Figures 2 and 3 provide evidence that the initial velocities of the reactions catalyzed by both fraction IV and V were proportional to enzyme concentration. The reaction mixtures containing fraction V (fig 3) showed high activity in the first 25 seconds and virtual inactivation after one minute. This phenomenon was observed regardless of enzyme or cytochrome c concentration. The evidence indicating the validity of assay used with this fraction is indicated in figure 3.

Heat Lability: Cytochrome oxidase in fraction IV (0 to 30 % ppt.) was completely inactivated by exposure for one minute in a boiling water bath but

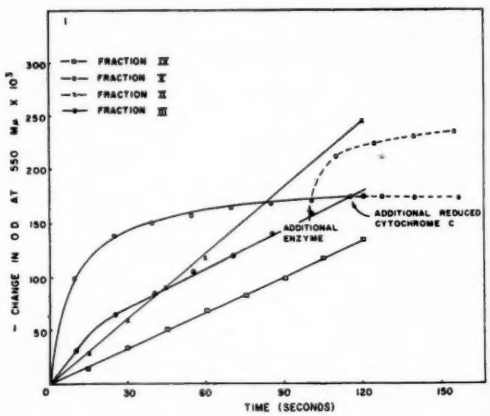
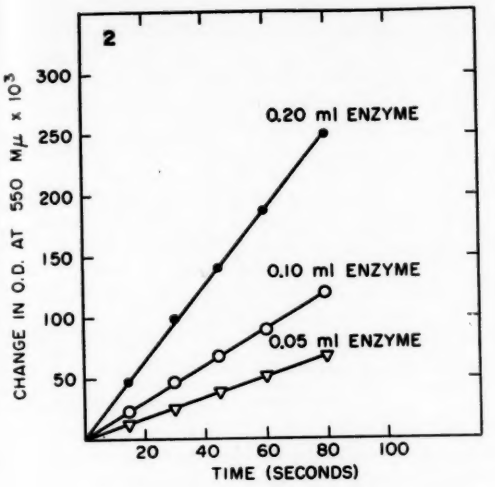


Fig. 1. Reaction kinetics of various cytochrome oxidase preparations. The standard assay procedure was used with the exception that reactions were allowed to proceed for the time periods indicated. The protein in mg added in fractions II, III, IV and V were respectively: 0.03, 0.02, 0.013 and 0.02. In the experiments involving fraction V two identical experiments were conducted for 100 seconds, however, since the curves were almost identical up to this point only one curve is shown. At 100 seconds additional enzyme (0.02 mg of fraction V) was added and the rising broken curve represents the course of the reaction. After 115 seconds in the other experiment involving fraction V additional cytochrome c (0.05 micromoles) was added as indicated. When additions were made O.D. changes were corrected for dilution.

The multiplication factor in the ordinate of this figure and in figures 2, 3, 4 and 5 applies to optical density change only.



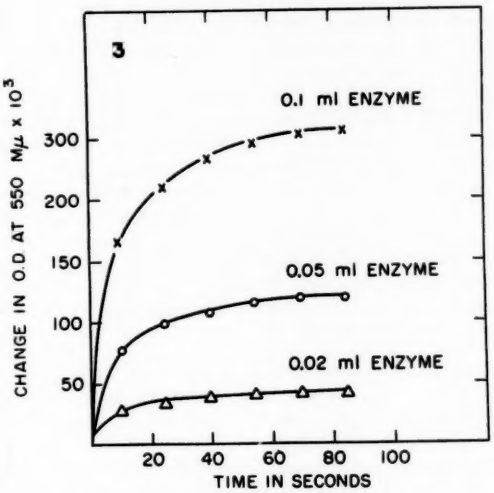


FIG. 2. Reaction kinetics of fraction IV at various enzyme concentrations. The standard assay procedure was used except for the time factor. The extract contained 0.27 mg of protein per ml.

FIG. 3. Reaction kinetics of fraction V at various enzyme concentrations. The standard assay procedure was used except for the time factor. The extract contained 0.30 mg of protein per ml.

the activity of fraction V decreased only 40 % under the same experimental conditions. Fraction V (30 to 65 % ppt.) however was inactivated by an exposure for five minutes in a boiling water bath. As previously indicated fraction III (figure 1) catalyzed a diphasic reaction. Since fractions IV and V were derived from fraction III (cholate extract of precipitated particulates) it was of interest to determine the heat lability of the latter. After exposure of fraction III to boiling water for one minute a reaction was catalyzed at a rate very similar to that indicated for fraction V in figure 1. This information indicates that two different factors were involved in the diphasic reaction catalyzed by fraction III. Fractions IV and V must then represent the two factors.

INHIBITORS: Experiments (fig 1) indicated that the cytochrome oxidizing factor in fraction V was inactivated by some substance and pointed to the cytochrome c preparation as the source of the inhibitor. Preincubation of fraction V with different concentrations of oxidized cytochrome c for various periods of time showed that the degree of inhibition was correlated with both of these variables (table II). Preincubation of the enzyme for five minutes with the cytochrome c concentrations ranging between 1.2×10^{-5} and 5×10^{-5} M resulted in complete inactivation in every case. The activity of fraction IV, on the other hand, was not appreciably affected by preincubation with oxidized cytochrome c at the various concentrations tested.

Since there was a possibility that the cytochrome c preparation contained some inhibitor, solutions of cytochrome c were prepared by reducing with either hydrogen and platinized asbestos or sodium hydrosulfite and these were dialyzed for 12 hours against three liters 0.05 M phosphate buffer at pH 7.0. Assays containing the dialyzed reduced cytochrome solutions still exhibited the characteristic inactivation when fraction V was utilized as the oxidizing factor but no inactivation was observed when fraction IV was utilized. The substitution in the assay medium of reduced cytochrome c prepared from pyrogen free cytochrome c (Sigma Chemical Co., St. Louis) resulted in the characteristic sharp decrease in activity after the initial 25 seconds. Several different preparations of both horse heart and beef heart

TABLE II

EFFECT OF OXIDIZED CYTOCHROME C ON THE ACTIVITY OF DISPERSED CYTOCHROME OXIDASE FROM SOYBEAN ROOTS

EXTRACT	CONCENTRATION OF OXIDIZED CYTOCHROME C	PREINCUBATION TIME WITH CYTOCHROME C (MINUTES)			
		0	1	3	5
	M	*Change in O.D. in 15 seconds at 550 mμ*			
Fraction IV	0.0	0.019	0.024	0.022	0.022
Fraction V	0.0	0.072	0.071	0.069	0.071
Fraction IV	1.2×10^{-5}	0.019	0.020	0.022	0.020
Fraction V	1.2×10^{-5}	0.063	0.025	0.020	0.000
Fraction IV	2.5×10^{-5}	0.020	0.020	0.021	0.021
Fraction V	2.5×10^{-5}	0.045	0.020	0.005	0.000
Fraction IV	5.0×10^{-5}	0.020	0.022	0.020	0.019
Fraction V	5.0×10^{-5}	0.038	0.015	0.000	0.000

The standard assay procedure was used. Fraction IV contained 0.08 mg and fraction V 0.02 mg of protein.

Fraction IV was prepared by 0 to 30 % $(NH_4)_2SO_4$ precipitation and fraction V by 30 to 65 % $(NH_4)_2SO_4$ precipitation.

cytochrome c from commercial sources were tested with the fraction V preparation and all behaved in a similar manner. This information suggests therefore that oxidized cytochrome c or an impurity in it inhibited the oxidation.

The possibility that the oxidizing factor was inactivated by the reduced form of cytochrome c as well as the oxidized form was investigated by using the leuco dye method outlined by Smith and Stotz (17). Fractions IV and V were found to actively catalyze the oxidation of reduced 2,3′,6-trichloroindophenol, ascorbate, and DPNH in the absence of cytochrome c. After treatment of fractions IV and V for one minute in a boiling water bath both extracts failed to catalyze the direct oxidation of ascorbate, DPNH, and leuco dye. Addition of cytochrome c to an assay medium containing leuco dye and the heat treated fraction V resulted in oxidation of the leuco dye. This cytochrome dependent leuco dye oxidation by fraction V was inhibited by the same compounds that were inhibitory to this preparation when measured by the standard procedure (table III). This cytochrome dependent leuco dye oxidation was not manifested with fraction IV extracts that had been exposed to boiling water. Catalytic concentrations of cytochrome c, however, increased the rate of leuco dye oxidation using the unboiled fraction IV. The rate of oxidation of leuco dye by the heat treated fraction V preparation decreased sharply with time (inset in fig 4). This curve is very similar to that indicated in figure 2 where reduced cytochrome c oxidation as catalyzed by fraction V was measured directly. The effect of various concentrations of cytochrome c on the enzyme activity measured by the leuco dye method is presented in the major graph of figure 4. Since there were considerably greater concentrations of leuco dye than cytochrome c in all reaction mixtures and since leuco dye and cytochrome c were mixed together before the enzyme was added, the reduced dye maintained the cytochrome c in the reduced state throughout the experimental period. Using this assay method, a concentration of 5×10^{-6} M cytochrome c resulted in the highest enzyme activity. Since higher concentrations of cytochrome c resulted in decreased enzyme activity, it seems probable that reduced as well as oxidized cytochrome c inhibited oxidation by fraction V. The possibility that the cytochrome c preparation contained an inhibitor as a contaminant, however, has not been ruled out.

Smith (16) reported that high concentrations of cholate inhibited cytochrome oxidase from animal sources. In an effort to determine whether the observed differences in the rates of reactions catalyzed

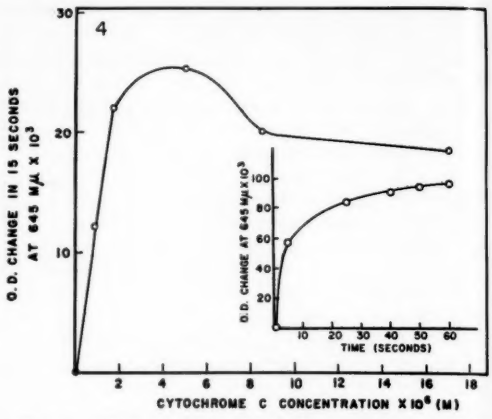


FIG. 4. (Major graph) Cytochrome oxidase activity at various concentrations of cytochrome c using the leuco dye method for assay. The assay medium in a final volume of 1 ml contained the following in micromoles: 0.5 of leuco dye; 50 of phosphate buffer at pH 7.0; and the concentrations of cytochrome c as indicated. The fraction V extract added to reaction mixtures was heated for one minute in a boiling water bath and contained 0.03 mg protein. Cytochrome oxidase activity was determined by the change in O.D. at 645 mμ in the 15-second interval between 10 and 25 seconds.

(Inset) Time course of the reaction using the leuco dye method for assay. The assay medium in a final volume of 1 ml contained the following in micromoles: 0.5 of leuco dye; 50 of phosphate buffer at pH 7.0; and 0.005 of cytochrome c. The amount and kind of extract added was identical to that indicated for the major graph of figure 4.

TABLE III

INHIBITION OF DISPERSED CYTOCHROME OXIDASE BY VARIOUS COMPOUNDS

INHIBITOR	CONCENTRATION	INHIBITION	
		FRACTION IV	FRACTION V
		%	%
NaN_3	10^{-3} M	78	0
NaN_3	10^{-2} M	97	7
KCN	10^{-3} M	89	1
KCN	10^{-2} M	92	33
CO (dark) *	19 to 1	62	..
CO (light) *	19 to 1	25	..
Antimycin A	5.5×10^{-6} M	0	35
Antimycin A	9.1×10^{-6} M	3	52

* Manometric assay utilized. All other tests involved the use of the standard spectrophotometric assay.

In all reaction mixtures except those involving CO the standard assay procedure was used after the enzyme in buffer was preincubated for 1 minute with the inhibitors indicated. These reaction mixtures contained 0.02 mg of protein from fraction IV and 0.01 mg of protein from fraction V.

For the determination of CO inhibition the procedure of Webster (21) was used with the exception that 75 micromoles of ascorbic acid was added to each flask instead of hydroquinone. In these experiments 2 mg of protein from fraction IV and 1.3 mg of protein from fraction V was included in the assays. Manometric assays were corrected for the oxidation in absence of added cytochrome c. Fraction IV was prepared by 0 to 30% $(NH_4)_2SO_4$ precipitation and fraction V by 30 to 65% $(NH_4)_2SO_4$ precipitation.

by fractions IV (0 to 30 % $(NH_4)_2SO_4$ ppt.) and V (30 to 65 % $(NH_4)_2SO_4$ ppt.) preparations were due to differential inhibition of the two fractions by cholate, samples of both fractions were dialyzed for 14 hours against each of the following cold solutions: 3 liters of 0.1 M phosphate buffer at pH 7.0; 3 liters of 0.02 M TRIS buffer at pH 7.0; and 3 liters of 0.1 M sucrose. Assays of the extracts after dialysis indicated that each contained concentrations of cholate of approximately 0.06 %. After the dialyses both extracts were assayed for cytochrome oxidase activity by the standard procedure. Fraction IV samples still catalyzed the oxidation of reduced cytochrome c at linear rates and fraction V extracts resulted in cytochrome c oxidation at the characteristic curvilinear rates. Extracts dialyzed against 13 % $(NH_4)_2SO_4$ solutions (18) behaved in a manner similar to those dialyzed against the buffer or sucrose solutions. The effect of Antimycin A, KCN and NaN_3 on cytochrome oxidase activity of fractions IV and V is shown in table III. Antimycin A had no effect upon fraction IV reactions but at low concentrations inhibited fraction V reactions markedly. KCN at 10^{-3} M almost completely inhibited activity of fraction IV. Oxidation by fraction V was insensitive to 10^{-3} M KCN and was inhibited only 33 % at 10^{-2} M KCN. Using the manometric assay it was demonstrated that fraction IV was inhibited by a 19 to 1 mixture of CO and oxygen and that the inhibition was markedly reversed by light. Since the reaction resulting from the fraction V preparation did not continue for periods greater than one minute, oxygen uptake by this fraction could not be demonstrated. It was shown by

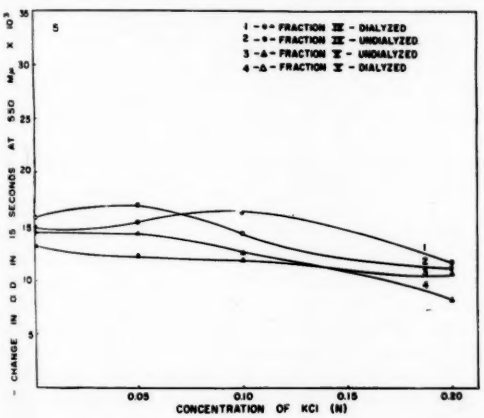

5
1 -●- FRACTION III - DIALYZED
2 -○- FRACTION III - UNDIALYZED
3 -▲- FRACTION V - UNDIALYZED
4 -△- FRACTION V - DIALYZED
CHANGE IN O D IN 15 SECONDS AT 550 mμ X 10³
CONCENTRATION OF KCl (M)
0.05
0.10
0.15
0.20

FIG. 5. Effect of various concentrations of KCl on cytochrome oxidase activity of cholate extracts from soybean roots. The procedure was that of the standard assay with variations in concentration of KCl as indicated. The quantities of protein in mg added to assays indicated by curves 1, 2, 3, and 4 are respectively: 0.04, 0.04, 0.025 and 0.05. Extracts used in reactions indicated by curves 1 and 2 were dialyzed for 14 hours against 3 liters of 0.02 M TRIS buffer at pH 7.4.

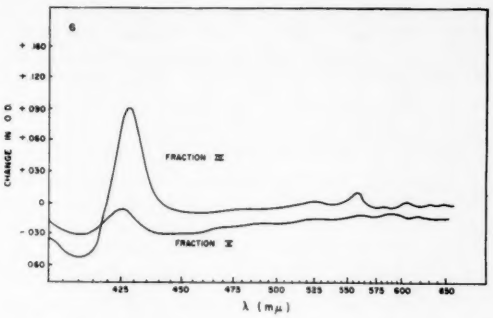


FIG. 6. Difference spectra (reduced minus oxidized) of cholate extracts from soybean roots. Fractions IV and V were prepared as indicated in Materials and Methods. To obtain the reduced minus oxidized difference spectra, sodium hydrosulfite was added to one cuvette and the spectra plotted within 90 seconds by use of a Warren Spectracord. Fractions IV and V contained 1.6 and 2.0 mg protein respectively.

use of a cuvette with a Thunberg attachment that a 19 to 1 CO-O_2 mixture did not inhibit the oxidation of reduced cytochrome c when fraction V was utilized as the enzyme. To date no oxygen requirement has been demonstrated for the reaction involving the fraction V preparation by use of a cuvette with a Thunberg attachment. Since the technique used was adequate for the demonstration of an oxygen requirement for cytochrome oxidase from other sources the question of involvement of oxygen in the fraction V reaction remains open.

EFFECT OF KCL ON ENZYME ACTIVITY: The activity of particulate cytochrome oxidase was markedly stimulated by salts as previously reported (11). The addition of KCl to assays containing either fractions IV (0 to 30 % $(NH_4)_2SO_4$ ppt.) or V (30 to 65 % $(NH_4)_2SO_4$ ppt.) failed to stimulate the activity. In order to remove salts from the preparations both fractions IV and V were dialyzed overnight against three liters of a solution of 0.1 M sucrose or an equal volume of a solution of 0.02 M TRIS buffer, pH 7.4 The cholate content was approximately 0.04 % after dialysis against sucrose. The cholate content of fraction V after dialysis against TRIS buffer was approximately 0.07 % and approximately 0.09 % in that dialyzed against sucrose. Sodium introduced into the reaction mixture was less than 2×10^{-3} M. Assay mixtures containing the dialyzed extracts showed no enzyme activation as a result of the addition of KCl at final concentrations ranging up to 0.2 M (fig 5). Both dispersed fractions, however, were inhibited by $NaHCO_3$ as previously reported for particulate preparations (10).

SPECTRA: Difference spectra of fractions IV and V in the range 400 to 620 mμ are shown in figure 6. The spectra were plotted with the oxidized pigments in the reference cuvette and dithionite reduced pigments in the 2nd cuvette. Peaks were observed in

the difference spectrum of fraction IV near 428, 527, 557 and 602 mμ. A peak at 426 mμ was evident for fraction V. Certain other preparations of fraction V exhibited additional peaks around 560 and 600 mμ, but the magnitudes of these peaks were much less than those observed with fraction IV. The difference spectrum of fraction III was similar to that of fraction IV. The oxidized extracts of all fractions absorbed strongly near 413 mμ.

Discussion

The purification of particulate cytochrome oxidase from soybean roots has been attempted in an effort to provide evidence of the mechanism of action specifically with reference to the mechanism whereby salts stimulate the enzyme activity in particulate extracts. Two dispersed fractions containing cytochrome oxidizing factors were prepared by use of sodium cholate.

The 1st preparation was obtained by precipitation of a protein fraction from the extracts of cholate treated particulates by adding $(NH_4)_2SO_4$ until the solution was 30 % saturated. The fraction possessed all the major characteristics which have been established for classical cytochrome oxidase (5, 7). The activity of this preparation was strongly inhibited by both KCN and NaN_3 and CO inhibition in the dark was reversed by light in manometric assays. Initial rates of the reactions catalyzed by this fraction approached linearity, an observation which is consistent with a previous report (11) with similar material. These results, however, are not in agreement with those obtained with the enzyme prepared from animal sources (18). Our assay system contained about three times the concentration of reduced cytochrome c used by other workers (2) and this may be an important factor contributing to the observed kinetics. A difference spectrum of the fraction IV (0 to 30 % $(NH_4)_2SO_4$ ppt.) preparation revealed evidence of reduced absorption bands characteristic of cytochrome oxidase and also evidence that cytochrome pigments other than the oxidase were contained in this extract. It seems clear that this preparation contained the classical cytochrome oxidase system. The extent of purification of the enzyme in the fraction was too small to be of appreciable significance.

The 2nd preparation referred to as fraction V and prepared by increasing the $(NH_4)_2SO_4$ concentration from 30 to 65 % saturation in a cholate extract of particulates behaved differently than that of fraction IV. Activity was not appreciably inhibited by KCN, NaN_3 or CO. It was inhibited, however, by a very low concentration of Antimycin A, a compound that had no effect on the fraction IV extract. It is well known (6, 13) that this antibiotic inhibits the step between cytochromes b and c, however, no similar inhibition between cytochrome c and oxygen has been reported. The difference spectrum of fraction V revealed no clear cut evidence of cytochrome bands with the exception of that near 426 mμ. Further purification will be necessary before the significance of this can be evaluated. Since reactions involving this extract ceased in a period usually less than one minute and since additional reduced cytochrome c failed to renew the oxidation (fig 1) the question of whether or not an enzyme was involved in the reaction is obviously presented. It was demonstrated that the oxidizing factor was non-dialyzable, labile to boiling for five minutes and required cytochrome c for the oxidation of reduced 2,3′,6-trichloroindophenol. Evidence is presented indicating that fraction V oxidation was inhibited by both oxidized and reduced cytochrome c. Smith and Conrad (18) have made similar observations with preparations from heart muscle. These properties certainly would not be expected if a non-enzymatic oxidation were involved. Since all efforts to demonstrate an oxygen requirement for the fraction V oxidation have failed, obviously the exact nature of the reaction remains for future investigation. It seems probable that the fraction V preparation represents some fragment of an electron transport system that has some peculiarly high affinity for cytochrome c. The possibility of preparatory procedures creating a fragment of a particle with an affinity for cytochrome c seems reasonable. It will be necessary to test the inhibition of the fraction V activity by highly purified cytochrome c preparations before it can be concluded with certainty that cytochrome c per se and not a contaminent is responsible for the inhibition.

It is of interest to consider that the cytochrome oxidase activity in particulate preparations is strikingly stimulated by salts but no such stimulation is observed with the cholate dispersed preparations. It would be logical to expect that sodium cholate would serve as a non-specific salt activator. Reaction mixtures including the dialyzed extracts containing approximately 2×10^{-3} M with respect to sodium cholate and 0.025 M with respect to TRIS chloride were not stimulated by the addition of KCl at concentrations ranging between 0.05 and 0.2 M. On the basis of previous studies (11) of the influence of salts on cytochrome oxidase activity in particulates, the combined sodium cholate and TRIS chloride concentrations in reactions involving the dispersed fractions would not have been sufficient for an appreciable salt stimulation. It seems possible that the stimulatory influence of salts on particulate cytochrome oxidase is manifested as a result of some physical effect on the enzyme that improves the accessibility of it to substrates and that the surface active properties associated with cholate salts make them highly effective in small concentrations. The possibility that an electrolyte may be required in the flow of electrons from one reactant to another in some structural unit of cells already has been discussed by Lundegardh (9) and briefly considered by Miller and Evans (11). From this viewpoint, it would seem logical that the destruction of the normal physical orientation within particulates by use of dispersing agents might allow direct interaction of reactants and therefore electron flow without a rela-

tively high electrolyte concentration. Apparently there is some specific effect of cholate salts not characteristic of other dispersing agents since Sisler (in preparation) has prepared by use of digitonin optically clear extracts of tobacco root particulates which contain cytochrome oxidase that is markedly stimulated by salts.

Summary

A dispersed preparation of cytochrome oxidase has been prepared by adding sodium cholate to particulates isolated from roots of soybean seedlings. By use of $(NH_4)_2SO_4$ the dispersed extract was fractionated into two parts. The addition of either fraction to a solution containing reduced cytochrome c resulted in rapid oxidation. The properties of the two fractions however differed greatly.

One fraction, which was obtained by 30 % saturation of the dispersed extract with $(NH_4)_2SO_4$, catalyzed reduced cytochrome c oxidation at a linear rate for a short period of time and exhibited all the well known properties of cytochrome oxidase that have been described for the enzyme from various sources.

The 2nd fraction, obtained by increasing the $(NH_4)SO_4$ saturation in the dispersed extract to 65 %, caused an oxidation of reduced cytochrome c at a curvilinear rate, then the reaction quickly ceased. The evidence presented indicates that oxidized cytochrome c or a contaminate in this preparation inhibited the oxidation and therefore is responsible for the inactivation. Also the activity of the 2nd fraction was strongly inhibited by Antimycin A but was not appreciably inhibited by NaN_3 or CO. These properties are in direct contrast to those exhibited by the 0 to 30 % $(NH_4)_2SO_4$ precipitated fraction. The 0 to 30 % $(NH_4)_2SO_4$ fraction catalyzed the uptake of oxygen; however, all efforts to demonstrate an oxygen requirement for the 30 to 65 % $(NH_4)_2SO_4$ fraction have failed.

Samples of the dispersed extracts were subjected to dialysis and afterward assayed for activities with and without added KCl. Concentrations of KCl that markedly stimulated the activity of particulate preparations had no appreciable effect on the dispersed fractions.

The results are discussed with respect to the properties of the two fractions and to the mechanism whereby salts activate particulate preparations and fail to activate cholate dispersed preparations.

The authors gratefully acknowledge the technical assistance of Mrs. Thea Schostag.

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SYSTEMIC INDUCTION OF SPROUTING IN WHITE POTATOES BY FOLIAR APPLICATIONS OF GIBBERELLIN[1,2,3]

L. F. LIPPERT, LAWRENCE RAPPAPORT AND HERMAN TIMM
DEPARTMENT OF VEGETABLE CROPS, UNIVERSITY OF CALIFORNIA, DAVIS, CALIFORNIA

Gibberellin influences the rest period or dormancy of seeds (4), shoots (1), and other plant parts (2, 5, 9). In potato, *Solanum tuberosum*, a condition of physiological rest prevails from the time of tuber initiation until 6 to 12 weeks after harvest depending on varietal characteristics (3). The rest period has been markedly curtailed by immersing freshly harvested potato tubers in gibberellin (9, 10). This effect, coupled with the rapid stimulation of growth of various plants by gibberellin (6), suggested that preharvest foliar sprays of this chemical might shorten the rest period of the immature, developing tubers. Results of the experiments described herein indicate that gibberellin initiates the sprouting process of the tubers on the plant when sprouting is least likely; they suggest further (7, 12) that gibberellin moves systemically in plants.

In a preliminary study (5) gibberellin applied as a foliar spray induced earlier sprouting of the subsequently harvested tubers. In a more detailed experiment, a spring crop of White Rose potatoes was grown in a split plot design with three replications of 20 plants per plot. The plants were sprayed to run-off four weeks, two weeks, or one week before harvest with solutions containing 0, 10, 50, 100, and 500 mg/l of gibberellin. At harvest, 111 days after planting, examination of all the resulting tubers revealed that gibberellin applied to the foliage as late as two weeks before harvest markedly stimulated sprouting (table I). This effect was most pronounced with the earlier applications and the higher concentrations. In contrast, tubers from the untreated plants showed little or no sprouting activity at harvest. Of the tubers from plants which received sprays containing more than 10 mg/l of gibberellin four and two weeks before harvest, some produced secondary tubers sessile to the main tubers or terminally on elongated sprouts (fig 1), others produced barely visible to prominent sprouts without secondary tubers, and the remaining tubers showed no sprouting activity.

TABLE I

PERCENTAGE * OF SPROUTED TUBERS AT HARVEST FROM PLANTS WHICH RECEIVED PRE-HARVEST FOLIAR SPRAYS OF GIBBERELLIN **

GIBBERELLIN MG/L	WEEKS BEFORE HARVEST		
	4	2	1
	(%)	(%)	(%)
0	0.0	1.4	0.2
10	3.0	1.5	1.5
50	58.3	18.0	0.4
100	75.6	34.3	2.1
500	83.6	50.0	5.8
		5%	1%
L.S.D. for time of application		6.3	N.S.
L.S.D. for concentrations		10.7	10.2
L.S.D. for interaction		N.S.	N.S.

* For statistical analysis original percentages were transformed to angles by using the formula:

$$\text{Angle} = \text{Arc Sin}\sqrt{\text{percentage}}.$$

** Average of three replications.

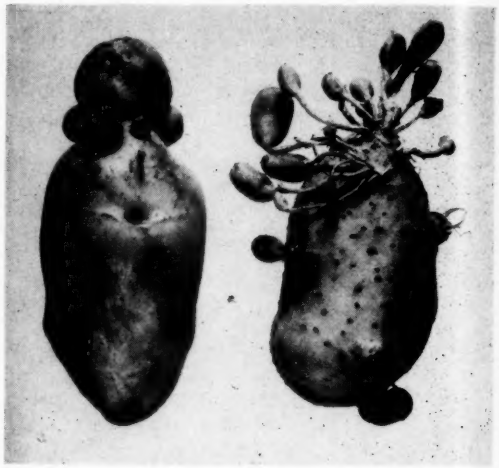

FIG. 1. The stimulation of sprouting and subsequent secondary tuber growth on potato tubers from plants sprayed 4 weeks before harvest with a solution containing 500 mg/l of gibberellin. (Photographed at harvest.)

Pre-harvest chemical sprays have inhibited sprouting (13), but the induction of sprouting in developing tubers by foliar-applied chemicals is unusual. That gibberellin is effective may be especially significant, since gibberellin-like substances occur in higher plants (8) and since gibberellin may be related to several important physiological processes (11).

The acceleration of sprouting by foliar sprays in this experiment was comparable to that obtained by immersing whole or cut tubers in gibberellin after harvest (9). The appearance of sprouts and secondary growth shortly after foliar-spray applications (table I) implies that gibberellin is translocated downward in potato plants.

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[2] Supported in part by a grant from Merck & Co., Inc., Rahway, New Jersey.
[3] The potassium salt of gibberellic acid was supplied by Merck & Co., Inc., Rahway, New Jersey.

Summary

Foliar applications of gibberellin four weeks, two weeks, or one week before harvest resulted in visible sprouting on developing potato tubers. This suggested the rapid translocation of gibberellin in potato plants.

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EFFECTS OF CHLORIDE AND SULFATE IONS ON THE GROWTH, LEAF BURN, COMPOSITION AND ANATOMICAL STRUCTURE OF TOBACCO (NICOTIANA TABACUM L.) [1,2]

WALTER F. WEDIN [3] AND B. ESTHER STRUCKMEYER

Department of Horticulture, University of Wisconsin, Madison 6, Wisconsin

The effects of chloride and sulfate ions on the growth, composition, quality and anatomical structure of plants are in need of clearer definition. Both anions are available in relative abundance to plants.

In addition to the growth and quality factors in most economic plants, the property of leaf burn in tobacco is very important. Satisfactory leaf burn, glowing capacity, or combustibility in cured tobacco must imply the ability to burn with a glow, thereby influencing aroma, flavor and character of the ash. Cellular structure has a bearing on texture, elasticity and other physical characteristics of the leaf which determine the general quality. Composition of the leaf influences the burn. It appears, therefore, that the leaf anatomy might also be related to the burn. Data presented elaborate on these varied effects.

Plant responses to Cl and SO_4 levels of supply have been subject to considerable study. Chlorine has been shown by Broyer (4) to be an essential element for plant growth. Beneficial effects on yield and quality of tobacco from moderate applications of Cl were reported by Garner et al (6). Many workers (2, 6, 13, 18) have found retardation in leaf burn due to Cl. The amount of Cl absorbed by tobacco is substantial (18).

Sulfur, known to be an essential element, was shown to benefit plant growth at lower levels of supply by Eaton (5). He also described the effects of excess SO_4 ion concentration and the wide variability in plant tolerances to these excesses. High applications of SO_4 have lowered leaf burn of tobacco (18, 19) but relative uptake of SO_4 was not as pronounced as Cl (14, 19).

The relation between anatomical structure and leaf burn of tobacco has not been defined. Behrens

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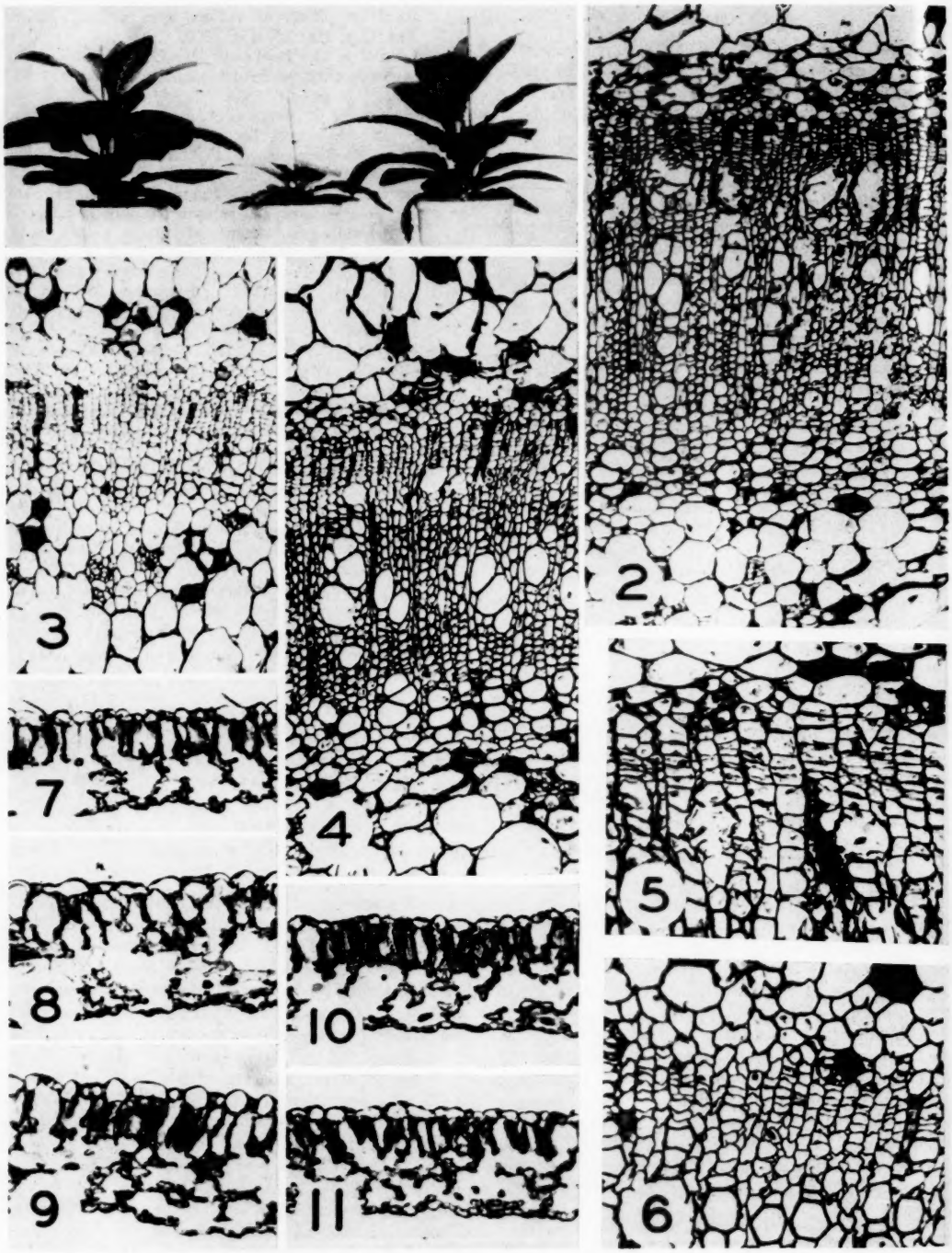
[3] Present address: Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, Minnesota.



FIGS. 1 to 11. Plants grown with complete, low SO_4 and low Cl nutrient solutions. Transverse sections of 6th internode from base of stem and leaves directly above 6th internode of tobacco 90 days after treatment.

FIG. 1. Left, complete nutrient solution; center, low SO_4; right, low Cl. FIG. 2. Stem from plant grown with complete nutrient solution. ×80. FIG. 3. Stem from plant grown with low SO_4. Less secondary vascular differen-

(3) stated that small, thick leaves with firm textures burned poorly. Johnson et al (13) found that poor burning leaves contained more of the heavier materials than did good burning leaves. However, they stated that structural relationships were difficult to separate from chemical relationships which could definitely influence leaf burn.

Materials and Methods

Tobacco (*Nicotiana tabacum* L.) of a cigar binder variety, Havana 307 was grown in quartz sand cultures at several levels of Cl and SO_4 ions in 1955 and 1956. Seeds were germinated in quartz sand and kept moist successively with distilled water, one fourth strength nutrient solution, one half strength nutrient solution and finally full strength nutrient solution when transplanted into large crocks with drains. Crocks with the various treatments were replicated on the greenhouse bench in a randomized complete block design.

Nutrient solutions prepared from distilled water and reagent grade salts varied in concentration. Composition of the nutrient solutions is shown in table I. The highest level of each ion was tested only in 1956. The basic solutions used were made up as described by Hoagland and Arnon (12) modified by the addition of NaCl and Na_2SO_4 to provide for the Cl and SO_4 ion levels. No special effort was made to exclude Cl and SO_4 ions completely, although a low level of each in the order of 1 ppm or less was maintained. Potassium levels were doubled in the 1956 tests to determine if the additional K would offset the Cl and SO_4 ion effect of lowering leaf burn. The plants received equal volumes of nutrient solution and distilled water on alternate days. Iron phosphate solution of 0.0005 % strength was supplied to the plants each week. The quartz sand in the crocks was leached weekly to prevent the accumulation of specific cations or anions in the crocks.

Leaf length measurements were taken on all leaves at 98 days of age in the 1955 studies and 112 days in the 1956 studies. The terminal growth of the plants (topping) was removed to a uniform size top leaf at 99 days of age in 1955. In 1956, plants were topped at 117 days of age. Leaf weights were recorded at the time of harvest for all leaves of each plant. The leaves were cured at room temperature and relative humidity of 35 to 40 % for 8 weeks.

Leaf burn tests were made according to the method described by Johnson et al (13). The average burn values presented represent a total of 6 ignitions for every leaf of the plant. Chemical determination of Cl was made according to the method described by Myrhre et al (18) and SO_4 was determined according to the A.O.A.C. (1) method.

Table I

Composition of Nutrient Solutions in ppm in 1956

Treatment	Cl	SO_4	N *	Na
Cl levels				
Low	1	192	280	1
35 ppm	35	192	280	23
70 ppm	70	192	280	46
140 ppm	140	192	280	92
280 ppm	280	192	280	184
SO_4 levels				
Low	1	1	336	1
48 ppm	1	48	336	12
96 ppm	1	96	336	23
192 ppm	1	192	336	46
384 ppm	1	384	336	92
Complete	1	192	336	1

Other major nutrients: K 234 ppm (1955), 429 (1956); P 31 ppm, Ca 200 ppm, Mg 49 ppm.

Minor elements: B 0.5 ppm, Mn 0.5 ppm, Zn 0.05 ppm, Cu 0.02 ppm, Mo 0.01 ppm, and Fe 1 ppm.

* N level 210 ppm (1955) in chlorine series and 266 ppm in SO_4 series.

In 1955, the 6th internode from the base of the plant and the leaf directly above this internode were sampled for anatomical examination 90 days after the start of the treatments. The samples were fixed in formalin aceto-alcohol and dehydrated with *n*-butyl alcohol. After the usual procedure of embedding, sections 10 μ in thickness were cut on a rotary microtome. The sections were stained with safranin O and fast green FCF.

Results

Growth: Plants grown with low SO_4 displayed characteristic sulfur deficiency symptoms; this being a lighter color in the young leaves and curling under of leaf tips as described earlier by McMurtrey (17). Plants from the 1955 studies at 100 days of age are shown in figure 1. Statistical analyses of the leaf length measurements revealed highly significant reductions in size due to the low SO_4 treatment in both years. The average measurements of all treatments are presented in figure 12.

Leaf weight recorded at harvest time in 1955 and 1956 tests are also presented in figure 12. The only treatment which caused a significant difference in leaf weight was the low SO_4 treatment.

Leaf Burn: Leaf burn values of tobacco grown at the several levels of Cl and SO_4 ion in 1955 and 1956 are presented in figure 12. The burn was not altered significantly by treatment in 1955. Differences among leaf burn values were highly significant in the 1956 studies. Treatments causing the significant differences were the Cl levels of 35 ppm, 140 ppm, and 280 ppm.

tiation and smaller cells. ×80. Fig. 4. Stem from plant grown with low Cl. Similar to plants grown with complete nutrient solution. ×80. Fig. 5. Cambial region, phloem and cortical cells of stem grown with complete nutrient solution. ×340. Fig. 6. Cambial region, phloem and cortical cells of stem grown with low SO_4. Cells smaller, less differentiation. ×340. Fig. 7. Leaf from plant grown with complete nutrient solution. ×165. Fig. 8. Leaf from plant grown with low SO_4. ×165. Fig. 9. Leaf from plant grown with 192 ppm SO_4. ×165. Fig. 10. Leaf from plant grown with low Cl. ×165. Fig. 11. Leaf from plant grown with 140 ppm Cl. Cells less compact than low Cl. ×165.

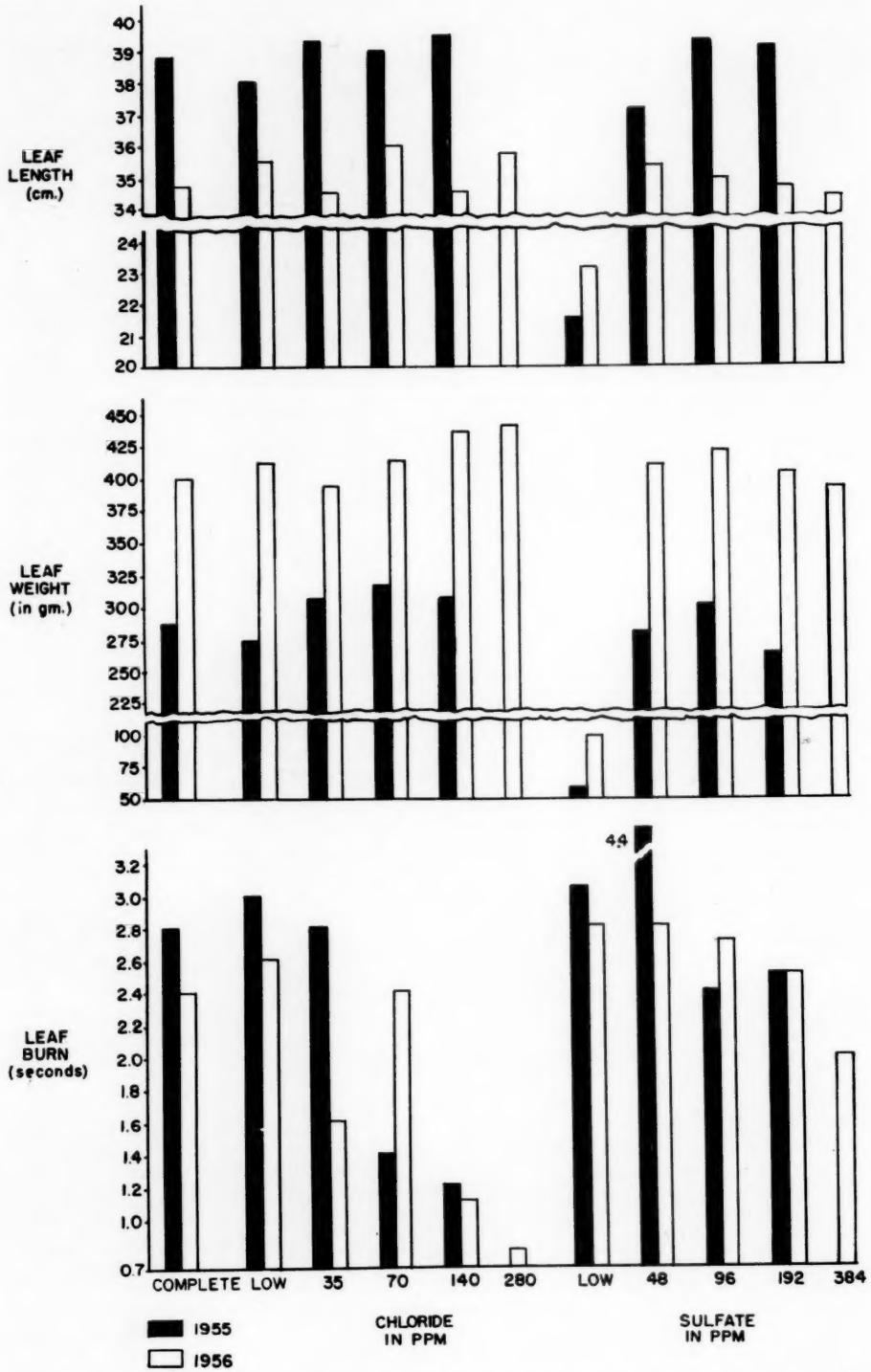


FIG. 12. Average length, weight and burn of tobacco leaves grown at various Cl and SO_4 nutrient levels in 1955 and 1956.

TABLE II

AVERAGE Cl AND SO_4 PERCENTAGES (OVEN DRY BASIS) OF TOBACCO LEAVES WHEN GROWN AT VARIOUS NUTRIENT LEVELS

TREATMENT	Cl		SO_4	
	1955	*1956*	*1955*	*1956*
Cl levels				
Low	0.12	0.12	...	1.93
35 ppm	0.83	0.85	...	1.64
70 ppm	1.53	1.27	...	1.58
140 ppm	2.86	2.33	...	1.66
280 ppm	...	2.94	...	1.30
SO_4 levels				
Low	...	0.29	0.32	0.32
48 ppm	...	0.17	1.11	0.90
96 ppm	...	0.14	1.41	1.16
192 ppm	...	0.17	1.66	1.39
384 ppm	...	0.15	...	1.60
Complete	...	0.12	2.40	1.42

CL AND SO_4 CONTENTS OF LEAVES: The percentages of Cl and SO_4 in leaves of tobacco grown in quartz sand culture are presented in table II. Complete analyses were not made in 1955 at all levels. However, the 1956 averages represent all treatments of Cl and SO_4 ion levels. Chlorine percentages in the tissue increased as the Cl ion nutrient level increased and decreased as the SO_4 ion nutrient level increased. Sulfate percentages in the leaves were increased due to increasing SO_4 ion concentration in the medium and decreases in leaf SO_4 resulted at the high Cl ion levels. The Cl percentage for tobacco grown with the complete nutrient solution was 0.12 %, the lowest leaf Cl value found excepting the low Cl treatment. Sulfate percentage for tobacco grown with the complete nutrient treatment (192 ppm SO_4) was 1.42 corresponding closely to the SO_4 percentage at the 192 ppm level in 1956. In the 1955 studies the SO_4 percentage of tobacco grown in the complete solution was higher and is unexplained on the basis of data available.

ANATOMY OF THE STEM AND LEAF: In 1955, samples taken from plants treated with low, 48, 96, and 192 ppm SO_4 and low, 35, 70 and 140 ppm Cl were examined. The 6th internode of plants grown with the complete nutrient solution (192 ppm SO_4) had an average diameter of 20.53 mm. Much of the tissue, including the interfascicular region, was composed of secondary vascular cells consisting of tracheids, xylem parenchyma and xylem vessels. An active cambium composed of 5 to 6 layers of cells was prominent. Phloem fibers had differentiated from both external and internal phloem. The cortical and pith cells were large and somewhat elongated in the tangential plane (fig 2, 5).

Stems of plants grown at low SO_4 had approximately half as much (11.97 mm in diameter) vascular tissue and smaller cell size when compared to the control. The xylem tissue was composed primarily of xylem vessels and some xylem parenchyma and the reduction in thickness of cell walls was striking. The epidermal, cortical and pith cells were smaller and rounder in shape than the controls. Three layers of cells comprised the cambium; phloem cells were smaller and phloem fibers failed to differentiate. Although there were no conspicuous areas of necrotic tissue, cells of the internal phloem and adjacent cells were sometimes misshapen and obliterated (fig 3, 6). Stems of plants grown with 48 ppm SO_4 continued to show a reduction in the amount of tissues, however, a trend toward a more normal cell structure was apparent. Cell size and shape was comparable to that of tobacco grown in the complete solution. With 96 ppm SO_4, little difference in anatomical structure could be observed from the complete. In 1956, plants were grown with 384 ppm SO_4 (double the amount of the complete nutrient solution) to determine if toxic effects could be induced. The amount of vascular tissue differentiated was similar to the control, however, cells were often misshapen, particularly those composing the vascular rays. The cortical cells were smaller and thinner walled, and the epidermis was not as distinct as the controls. The shape of the cambial cells was less discrete than the normal.

The striking differences in anatomical structure of plants grown with low SO_4 as compared to those grown in the complete nutrient solution were not observed within the Cl series (fig 4). Cellular abnormalities such as collapse and obliteration of cells were not present. Compared to the control plants, those grown with low Cl showed a very slight reduction in amount of vascular tissue. With higher concentrations of Cl (140, 280 ppm) some reduction in secondary vascular tissue occurred, particularly in the interfascicular region. With 280 ppm Cl, there were fewer layers of cortical cells, and smaller but thicker walled secondary xylem elements. The diameter of the stem was nearly comparable to the control plants, however, the pith area was larger.

The leaves from plants grown with low and high concentrations of SO_4 and Cl were thicker than the control in both years. The cellular structure of leaves from low SO_4 plants showed fewer cells giving a more open aspect compared to the more compact layer of palisade cells grown at 192 ppm (fig 7, 8, 9). Plants grown with low Cl showed a greater compactness in the palisade layer than those grown at 240 ppm, which was the reverse of what was apparent in the low SO_4 leaves (fig 10, 11).

DISCUSSION

Although S deficiency symptoms were manifest under conditions of this experiment, visual Cl deficiency symptoms were not evident. Admittedly, the techniques used in these nutrient studies were inferior to those required to obtain Cl deficiency. As described by Ulrich and Ohki (21), failure to obtain Cl deficiency symptoms in many cases may be due to air and salt contamination. These may have been major sources of contamination in the present investigations. Despite the failure to obtain visual Cl deficiency symptoms in these tests there were interesting factors affected by the high levels of Cl supplied.

Deviations in leaf length and weight in the Cl series were not marked. Growth was greater, how-

ever, as Cl concentration increased. The highest value recorded for leaf weight in the Cl series was noted for the 280 ppm level.

Striking effects on leaf length and green weight of leaves were found in the SO_4 series. Tobacco grown at the 96 ppm level had the greatest leaf length. Successive increases to the 384 ppm concentration were associated with reductions in average length of leaf. Leaf-weight values, highest at 96 ppm SO_4, decreased at the higher concentrations of SO_4. Average leaf weight of tobacco grown in the complete solution was comparable to the 192 ppm SO_4 level.

The reduction in leaf length and weight at the higher SO_4 levels suggests that an osmotic effect of the nutrient solution might be a factor. Hoagland (10) found that solutions of 2.0 to 2.5 atmospheres might exert inhibitive effects on plant growth. However, approximate calculations of pressure in solutions of this experiment are less than the inhibitive level. Although the Na ion was added at several levels in the present tests, it may not have been exerting inhibitive effects. McEvoy (16) found in extensive tests that yields of flue-cured tobacco grown in sand culture were not affected significantly by Na supply.

Furthermore, growth reductions in tobacco associated with the high SO_4 supply appear to be in contrast to the findings of McEvoy (15). He reported a wide range of tolerance to the SO_4 ion. It must be stated here, however, that data from this experiment does not include cation uptake. The addition of both Na and SO_4 may have caused wide fluctuations in the Na uptake of the plants and thus affected materially the entire cation-anion relationship and growth.

Inhibitive effects of increased Cl concentration on leaf burn were clearly demonstrated in these tests. Actual reduction at higher levels was marked in both years of testing. Effects of SO_4 levels on leaf burn were not as marked. In fact, the over-all leaf burn values in the SO_4 series were generally higher than at comparable concentrations of the Cl ion. Sulfate ions at the higher levels decreased leaf burn in these tests thereby verifying findings of other workers.

The contents of Cl and SO_4 in the tissue as influenced by levels in the nutrient medium substantiate previous findings of other workers. Sulfate sulfur uptake was depressed by an increase in Cl level and similarly Cl uptake was depressed by increased SO_4 in the medium. Hoagland (11) has discussed this relationship of Cl and SO_4 levels and their bearing on uptake by the plant. Data obtained from the present tests and those of Hoagland do not agree with those of Reisenauer and Colwell (20) who reported that a high rate of S application significantly increased concentration of Cl in the leaf. Gauch and Eaton (7) stated that increasing the SO_4 concentration of the solution decreased Cl absorption while an increase in Cl concentration did not affect SO_4 percentages in the plant. Thus, it appears that considerable disparity of opinion still exists with reference to the influence of the Cl and SO_4 ion. It may be dependent, as Eaton (5) has stated, on the cations that are present and the tolerance of different plants to the Cl and SO_4 ion.

The relative increases in Cl and SO_4 percentages in tissue in proportion to the concentration in the medium were of interest. An increase in the Cl ion level from 35 ppm to 280 ppm caused an increase from 0.85 % to 2.94 %. A corresponding sevenfold increase of the SO_4 ion from 48 ppm to 384 ppm only increased the percentage of SO_4 from 0.90 to 1.60 in the tissue.

The striking effects of low SO_4 as shown in these studies were practically corrected at the 96 ppm SO_4 level. It would appear that the minimum SO_4 level needed from the standpoint of normal structure as well as growth and leaf burn would be between 96 and 192 ppm. Reduced growth and leaf burn and misshapen cells with smaller, thinner walls were evident at the high SO_4 level of 384 ppm.

Thickening in the secondary xylem elements at the 280 ppm Cl level as found in these tests are in agreement with the findings of Hayward and Long (9) for the tomato where high concentrations of NaCl were supplied in the substrate. Increase in leaf thickness of plants grown at high levels of Cl and SO_4 in the present tests also agree with results reported by Hayward and Long (8).

Anatomical structure of the leaves did not reveal a reason for better leaf burn of tobacco grown at low concentrations of SO_4 or Cl ion. This also agrees with earlier results reported by Johnson et al (13) who found no significant difference in the anatomy of leaves with good and poor burn quality.

Summary

1. Tobacco plants grown in quartz sand culture at low SO_4 exhibited characteristic sulfur deficiency symptoms.

2. Statistical analyses of average leaf length revealed highly significant reductions in size due to the low SO_4.

3. Average length and weight of leaves decreased as SO_4 levels were increased beyond 96 ppm.

4. Plants grown at low Cl exhibited no marked growth responses but increases in Cl to 280 ppm caused moderate increases in average length and weight of leaf.

5. Inhibitive effects of high Cl on leaf burn were clearly demonstrated while effects of high SO_4 were not as marked.

6. Effects of Cl and SO_4 ions on chlorine and sulfate sulfur percentage in the leaf were shown. Chlorine percentage in the leaf was increased to a greater extent than sulfate sulfur by additions of the respective ion to the substrate.

7. Abnormalities in the stem structure were found with low SO_4. Increased leaf thickness was found at higher concentrations of the Cl and SO_4 ion.

8. Variations in burn of tobacco leaves could not be explained on the basis of anatomical structure.

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MULTIPLE SITE UPTAKE OF INDIVIDUAL CATIONS BY ROOTS AS AFFECTED BY HYDROGEN ION[1]

MAURICE FRIED and J. C. NOGGLE

Soil and Water Conservation Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland

Many investigators have suggested that metabolic uptake of ions by cells involves a system of carriers. A comprehensive review is given by Epstein (6). The ion combines with the carrier to form an active intermediate. The active intermediate breaks down essentially irreversibly to release the ion inside the cell.

Cations that are similar chemically may compete with each other for the same binding site. However different carriers are involved for different groups of cations. Thus Collander (4), and Epstein and Hagen (7), and Menzel and Heald (19), have indicated that rubidium and cesium and potassium are competitive for the same site. Although calcium utilizes a different site than potassium, Epstein and Leggett (8), and Menzel and Heald (19) have shown that strontium and barium compete with calcium. Neither sodium nor magnesium was found to be competitive for either of these sites.

Over a limited concentration range Epstein and Hagen (7), and Epstein and Leggett (8) noted one carrier site for each cation. Epstein and Hagen noted an anomaly at higher ion concentrations with sodium and Epstein and Leggett an anomaly with strontium. Over a wide concentration range Hagen and Hopkins (12) demonstrated two carrier sites for phosphate uptake.

Various investigators (14, 15, 16, 21) have shown that the hydrogen ion concentration of the bathing medium affects ion uptake. Others (1, 2, 3, 11) have minimized this effect within usual physiological ranges. Jacobsen et al (17), using the model of Ro-

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senberg (22), suggested that the cation carrier is metabolically produced at the external surfaces as the hydrogen complex, HR, and that the anion carriers were metabolically produced at the external surfaces as the hydroxyl complex R'OH. They formalized this in equations a and b,

$$HR + M^+ \rightleftharpoons MR + H^+ \quad (a)$$
$$R'OH + A^- \rightleftharpoons R'A + OH^- \quad (b)$$

where HR and R'OH represent the metabolically produced cationic and anionic binding substances. MR and R'A serve as transporting agents, transferring ions from culture medium to the vacuole (17).

On the other hand, Epstein and Hagen (7) and Hagen and Hopkins (12) have described ion uptake by carriers without any detailed assumptions as to the nature of the carrier. Ion absorption is described by these authors in the following equations.

$$R + M_{(outside)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MR \quad (1)$$

$$MR \underset{k_4}{\overset{k_3}{\rightleftharpoons}} R' + M_{(inside)} \quad (2)$$

where R represents a metabolically produced site, M, the ion, MR, the active intermediate and k the rate constant for each reaction.

Hagen and Hopkins (12) have shown by kinetic analysis that hydroxyl ion is a competitive inhibitor of phosphorus uptake and behaves similarly to other competitive inhibitors such as ADP and pyrophosphate in phosphate uptake (13) and selenate in sulfate uptake (18).

The competitive inhibition of phosphate uptake by hydroxyl ion is consistent with the uptake mechanism described by both Hagen and Hopkins (12) and Jacobsen et al (17). However, the present authors prefer the description afforded in equations 1 and 2 since it makes no assumptions as to the location of the carrier nor the specific combination in which the carrier occurs. By analogous interpretation from kinetic analysis as developed for ion absorption (7, 12), the present results demonstrate that hydrogen ion is a competitive inhibitor of cation uptake. The cations used were sodium, potassium, rubidium and strontium. Although different sites are involved for various cations, two sites are shown to be involved in uptake of each of these cations. Competitive inhibition is demonstrated at one of the two sites of ion uptake found for each cation. This is the site that dominates ion uptake at lower ion concentrations. Although hydrogen ion effects are also demonstrated at higher ion concentrations where a second site dominates cation uptake the experiments were not designed to show whether this effect is competitive.

Methods

Theory: Where k_3 in equation 2 is rate limiting in the forward direction and k_4 is negligible, the observed absorption of an ion is proportional to MR as shown in equation 3.

$$v = k_3 [MR] t \quad (3)$$

where v = the amount of ion, M, absorbed in time, t.

From these equations a velocity equation can be derived analogous to case III for enzyme action described by Hofstee (16) and utilized by Hagen, Leggett and Jackson (13) for phosphorus uptake.

$$v = -K_m \frac{v}{[M]} + V_{max} \quad (4)$$

where K_m equals $\frac{k_2 + k_3}{k_1}$ analogous to the Michaelis constant (20), and V_{max} equals the amount absorbed when all of the carrier is saturated with the ion under study, i.e., MR = ΣR.

If v is plotted against $\frac{v}{[M]}$ a straight line should result for each site provided [M] remains essentially constant during the steady state uptake. The slope of this straight line is $-K_m$, the apparent dissociation constant of MR, when $k_2 \gg k_3$ (16). The y intercept is V_{max} and $V_{max} = k_3 [\Sigma R] t$.

If another ion competes for the same site, the V_{max} is the same in the presence of the competing ion (12) but the slope of the line changes. This change in slope reflects a decrease in the amount of ion at a given concentration associated with the site in the presence of a competing ion. Although the presence of one ion may affect the rate of uptake of another ion without being competitive, such an effect always results in a changed V_{max} and may also result in a change in slope.

It is more convenient to determine the total ion sorbed (absorbed plus adsorbed) instead of the total absorbed. Since adsorption not involved in uptake is negligible for monovalent cations as shown by Fried, Noggle and Hagen (10) and for phosphate as shown by Hagen and Hopkins (12) this would change equation 3 to 3a below:

$$v = [MR] k_3 t + [MR] \quad (3a)$$

However it would not affect equation 4 except that

$$V_{max} = [\Sigma R] k_3 t + [\Sigma R] \text{ instead of } V_{max} = [\Sigma R] k_3 t \quad (3b)$$

Thus, whether amount of ion absorbed or total sorbed is measured a plot of v vs $\frac{v}{[M]}$ will give a slope, $-k_m$, the apparent dissociation constant.

Technique: The methods employed are similar to those described by Epstein and Hagen (7). Seeds of *Hordeum vulgare*, var. Atlas 46, were germinated in demineralized water and grown for 6 days in continuously aerated 2×10^{-4} M $CaSO_4$. Twelve hours before an experiment was to begin, the roots were rinsed and demineralized water was substituted for the $CaSO_4$ solution. The roots were excised, rinsed and suspended in two liters of continuously aerated,

demineralized water just prior to making the sorption studies.

Chloride salts of Rb, Na, K and Sr were labeled with Rb^{86}, Na^{24}, Na^{22}, K^{42}, and Sr^{89} respectively. The pH of Rb and K solutions were adjusted to the desired value with 0.1 *N* NaOH or 0.1 *N* HCl. The pH of Na solution was adjusted to desired value with 0.1 *N* KOH or 0.1 *N* HCl.

The excised roots were blotted on washed, dry cheesecloth. One half gram portions of roots were weighed for Rb, K, or Na studies and 1.5 g portions for Sr studies and placed in beakers of continuously aerated water. The water was decanted and replaced by the appropriate radioactive solution to begin the sorption period. The cation concentration was maintained essentially constant by proper selection of root : volume ratios. At the end of the sorption period for Rb, Na or K, the solution was decanted and the roots were rinsed four times with demineralized water to remove adhering solutions and "outer space" (5) ions. The ion still associated with the root was considered total ion sorbed. At the end of the sorption period for Sr the solution was decanted, roots were rinsed with demineralized water, and adhering ions desorbed for a period of 30 minutes in continuously aerated solution of nonradioactive 10^{-2} M $CaCl_2$. The Sr still associated with the root was considered total Sr absorbed. After rinsing, the roots were placed into circular, ¼-ounce seamless tin boxes and dried under an infrared lamp prior to measurement of activity. Aliquots of the radioactive solutions were pipetted into tin boxes, dried, measured and used as a standard for computing ion absorption by roots.

Experimental Results

The uptake of cation in a given period of time was plotted against the uptake divided by the concentration of cation in solution. The times chosen were in the steady state region as shown by Fried, Noggle and Hagen (10). This was 20 minutes for rubidium and potassium, 30 minutes for sodium and one hour for strontium.

The uptake of rubidium at various concentrations from 10^{-6} to 10^{-2} M was measured in the presence and absence of 10^{-5} and 10^{-4} M potassium. The results are presented in figure 1.

One site, assuming the carrier concept of ion uptake, should give a straight line relationship. However a curve was obtained. Since the curved line can be resolved into at least two linear components a and b, by the use of polar coordinates more than one site must be involved in rubidium uptake. Because the relationship appeared to approach linearity at both extremes of the curve, the linear components can also be approximated by extrapolation to the intercepts at both coordinates (fig 1). This approximation is feasible because the apparent K_m for each component differs widely (12). Therefore, a likely explanation for the experimentally observed phenomenon was

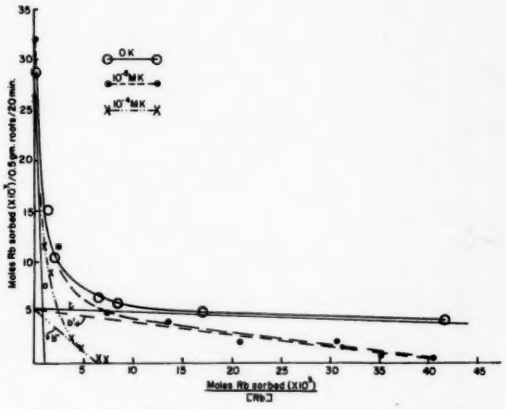

FIG. 1. The effect of potassium concentration on the uptake of rubidium by 0.5 g (fresh wt) of excised barley roots in 20 minutes. The uptake from solutions of various RbCl concentrations is plotted as a function of uptake divided by Rb concentration. The b-component of the reactions is separated at all potassium concentrations and the a-component at the zero-k level.

that two different sites were operative for rubidium absorption, one dominant at lower concentrations of the ion and the other at higher concentrations.

The uptake of rubidium in the presence of different concentrations of potassium also gives curved lines when plotted in the same manner. Again these curves can be separated into two linear components. The linear component at lower concentration of rubidium are illustrated by lines b′ and b″. These linear components have the same V_{max} as the linear component, b, in the absence of potassium. This indicates that the decrease in the uptake of rubidium represents direct competition by potassium. Apparently potassium combines with the same carrier as rubidium.

The uptake of sodium, potassium, rubidium and strontium at various concentrations was measured in presence of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M hydrogen ion. Hydrogen ion concentration was maintained constant during the sorption period. The uptake of ion was then plotted against the uptake divided by concentration. For the sake of simplicity only the results at pH 4 and 6 are shown. At these pH levels no adjustment of pH was necessary during the experiment. The results at pH 5 were intermediate and those at pH 7 did not differ appreciably from pH 6 except for sodium which continued to show a hydrogen ion effect at the higher pH levels. For rubidium, potassium and sodium the total ion sorbed was measured. For strontium only the total ion absorbed was measured. Results are presented in figures 2 a, b, c, and d.

Again curved lines were obtained at all pH levels. The two linear components were again approximated by extrapolation to the intercept of both coordinates.

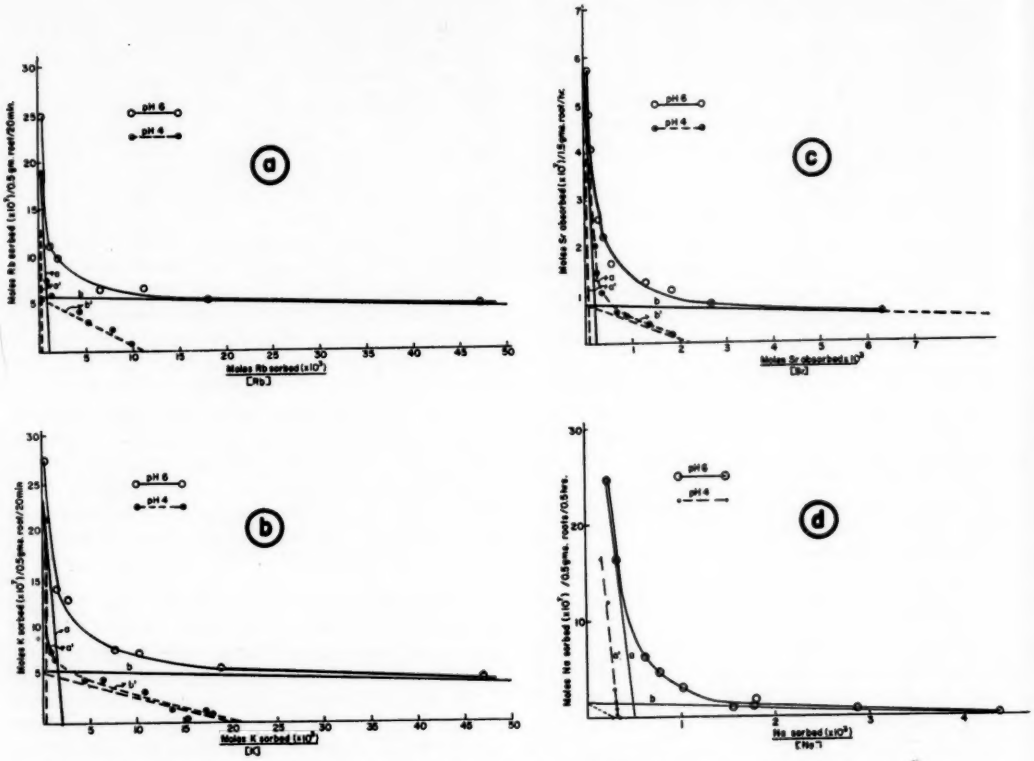

FIG. 2 a–d. The effect of pH on the uptake of cations by excised barley roots. The uptake from various concentrations of the cation is plotted as a function of uptake divided by concentration. The component reactions are separated into lines a and b for pH 6 and a′ and b′ for pH 4. Fig. a. Moles of Rb sorbed ($\times 10^7$) by 0.5 g (fresh wt) of roots in 20 minutes. Fig. b. Moles of K sorbed ($\times 10^7$) by 0.5 g (fresh wt) of roots in 20 minutes. Fig. c. Moles of Sr absorbed ($\times 10^7$) by 1.5 g (fresh wt) of roots in 60 minutes. Fig. d. Moles of Na sorbed ($\times 10^7$) by 0.5 g (fresh wt) of roots in 30 minutes.

Apparently the uptake of the four cations studied takes place from at least two distinct sites for each of the cations.

The results recorded in figure 2 also show that hydrogen ion is strictly competitive with each of the cations for b, the site that dominates ion uptake at low concentrations of the cation and probably competitive for the other site, a, that dominates ion uptake at the higher cation concentrations. The strict competition of hydrogen ion is evident because the V_{max} (y intercept) is the same at both pH levels and the slope of the component lines differs at the two pH levels.

DISCUSSION

The uptake of each cation, apparently involves at least two distinct sites. This is somewhat analogous to the finding that two sites are involved in phosphorus uptake by excised barley roots as described by Hagen and Hopkins (12). However, the two sites for phosphorus were shown to be related to two separate ion species, $HPO_4^=$ and $H_2PO_4^-$. No such explanation is apparent for cations, particularly monovalent cations.

At least two sites of ion uptake were demonstrated for strontium, rubidium, potassium and sodium. Other cations may also have multiple sites. The potassium sites may be identical to the rubidium sites as suggested by figure 1 and the work of Epstein and Hagen (7). The calcium sites may also be identical to the strontium sites as indicated by both Epstein and Leggett (8) and Menzel and Heald (19) at relatively high ion concentrations.

The slope of line a and b equal the respective K_m values, the apparent dissociation constant of MR (the active intermediate as discussed under "METHODS"). These K_m values were calculated for Rb, K and Sr. The results recorded in table I under the heading K_m are a mean of several experiments. The results indicate that potassium, rubidium, and strontium carrier complexes have approximately the same apparent dissociation constants.

To calculate the dissociation constant, K_1, of the complex formed with the competing ion, requires a

TABLE I

APPARENT DISSOCIATION CONSTANTS OF Na, K, Rb, AND Sr AND THE APPARENT DISSOCIATION CONSTANTS OF COMPETING IONS

CATION SORBED	SITE DESIGNATION	APPARENT DISSOCIATION CONSTANTS		
		K_m	K_i (HYDROGEN ION)	K_i (POTASSIUM)
Na	a	4×10^{-3}		
Na	b	1×10^{-5}	2×10^{-6}	
K	a	1×10^{-3}		
K	b	3×10^{-6}	1×10^{-5}	
Rb	a	2×10^{-3}		
Rb	b	4×10^{-6}	1×10^{-5}	4×10^{-6}
Sr	a	3×10^{-3}		
Sr	b	3×10^{-6}	1×10^{-5}	

generalized relationship for uptake in the presence and absence of the competing ion.

The equation for a competing ion used by Hagen and Hopkins (12) and Epstein and Hagen (7) when operating in the same system for the ion under test is formally analogous to equation 1 and is shown below as equation 5

$$I + R \rightleftharpoons IR \qquad (5)$$

where R in equation 1 and 5 is the same carrier, I is the competing ion and IR a complex of the competing ion and the carrier. By considering the equations for the equilibrium constants of the two reactions and the conservation equation

$$[\Sigma R] = [R] + [IR] + [MR] \qquad (6)$$

a mass action equation can be derived relating the concentration of the ions and the apparent dissociation constants to the amount of active intermediates.

$$[MR] = [\Sigma R] - \left(K_m + \frac{[K_m][I]}{K_i}\right)\frac{[MR]}{[M]} \qquad (7)$$

By substituting for MR and ΣR the respective relationships obtained from kinetic equations of steady state, 3a and 3b, equation 8 is obtained:

$$v = V_{max} - \left(K_m + \frac{[K_m][I]}{K_i}\right)\frac{v}{[M]} \qquad (8)$$

where a plot of v vs v/[M] has a slope =

$$-\left(K_m + \frac{[K_m][I]}{K_i}\right).$$

Thus the apparent dissociation constant for potassium at the site that dominates at lower rubidium concentrations can be calculated from slope of line b (K_m), and either b′ or b″, $-\left(K_m + \frac{[K_m][I]}{K_i}\right)$ in figure 1. The apparent dissociation constants for hydrogen ion at the sites dominant at lower ion concentrations of rubidium; potassium and strontium sites can also be calculated from the appropriate lines b, pH 6 and b′, pH 4 in figure 2. Each run gave a slightly different value. The averages of several determinations are presented in table I.

The apparent dissociation constant, K_m, of potassium as determined by the uptake of potassium was approximately the same as the apparent dissociation constant of potassium, K_i, from the rubidium site. Again it appears likely that the rubidium site b and potassium site b are one and the same site.

The apparent dissociation constant of the hydrogen ion complex, K_i, at site b is approximately the same for the site complexes of the three cations K, Rb, and Sr. This is higher than the dissociation constants for the cations themselves but only by a factor of approximately three. The magnitudes of the dissociation constants indicate that both hydrogen ion and the cations are relatively tightly held.

The K_i (hydrogen ion) for sodium could not be calculated from data in figure 2 d. A 10^{-4} molar solution of hydrogen ion almost eliminated the appearance of site b, figure 2 d, in the sodium uptake system, indicating that hydrogen ion was more tightly held than sodium at this site. The same hydrogen ion concentration also had a marked effect on site a. Since hydrogen ion had a greater affinity for the sodium site than did sodium, pH 6 was not adequate to eliminate the effect of hydrogen ion at lower concentrations of sodium.

The K_m for a cation must be calculated in a system in which the concentration of competing ion is negligible. The K_m for sodium was therefore calculated from uptake data at pH 7. The K_i (hydrogen ion) was calculated from comparative uptake data at pH 5.0. The results are recorded in table I.

The K_m for sodium at the site that dominates at lower concentrations appears to be somewhat greater than that for the other cations tested. This indicates a slightly lower affinity of sodium for the sodium site than of the other cations for their respective sites. The K_m of site a is similar to that found for the other cations. The K_i (hydrogen ion) is smaller for sodium than the K_m of sodium indicating again that hydrogen is more tightly held than sodium. Hydrogen ion also appears to be more tightly held to the sodium sites than to the other cation sites.

The hydrogen ion competes with rubidium, potassium, sodium and strontium, and possibly most other cations. However, the dissociation constants for hydrogen ion on the b site are of same order of magnitude as the dissociation constants for the cation. Thus, when cation concentration is appreciably higher than hydrogen ion concentration there is little, if any, pH effect due to competition. However, if the concentrations are similar, marked pH effects may be noted.

SUMMARY

The uptake of rubidium, potassium, sodium and strontium by excised barley roots under steady state conditions indicates at least two distinct carrier sites of absorption for each of the cations. One site domi-

nates at higher ion concentrations while the second dominates at lower ion concentrations.

The apparent dissociation constants were determined at each of the two sites for each of the four cations studied. They were similar in magnitude for potassium, rubidium and strontium and slightly higher for sodium.

Hydrogen ion was shown to compete directly for each of the sites that dominates at lower concentrations of all cations tested. The apparent dissociation constant for hydrogen ion at the strontium, rubidium and potassium site that dominates at lower concentrations was 1×10^{-5}. The comparable dissociation constant, K_1, at the sodium site was 2×10^{-6}. The apparent dissociation constants for hydrogen were similar in magnitude to the apparent dissociation constants of the ions themselves. Rubidium and potassium were shown to compete for the same site.

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GROWTH SUBSTANCES IN CORN POLLEN[1,2,3]

H. N. FUKUI, F. G. TEUBNER, S. H. WITTWER and H. M. SELL

Departments of Agricultural Chemistry and Horticulture,
Michigan State University, East Lansing, Michigan

Growth substances have been detected in the pollen of apple (3), orchid (7), tobacco (5), hazelnut shrub and pine (14), and corn (6, 10, 13). Studies of the growth substances in corn pollen have been concerned solely with crude ether soluble extracts. This communication describes the growth substances found in the acid and non-acidic or "neutral" fractions in ethyl ether extracts of corn pollen, *Zea mays*, var. Golden Cross.

Freshly harvested pollen (166 grams) was extracted with cold, peroxide-free ethyl ether at 2° C for four hours. The supernatant was decanted and extracted with 5 % aqueous sodium bicarbonate. The

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non-acidic or "neutral" substances remained in the ether layer, while the acidic substances passed into the bicarbonate layer. The bicarbonate solution was acidified with hydrochloric acid to pH 2.8 and extracted with ethyl ether. This ethereal solution contained the acidic substances. The acidic and "neutral" solutions were separately concentrated to 2 ml under reduced pressure with the bath temperature kept below 25° C. The concentrated "neutral" oil-like residue was then partitioned between acetonitrile and hexane (8). The hexane layer containing the lipids and some of the pigments was discarded, while the acetonitrile layer was concentrated under reduced pressure. The concentrated extracts were partitioned by paper chromatographic technique on Whatman No. 1 filter paper with a solvent mixture composed of 2-PrOH : NH_3 : H_2O (8 : 1 : 1 v/v) (12) and by water (11). The dried chromatograms, each representing 8 g of pollen, were scanned for ultraviolet fluorescent spots with a 2537 "Mineralight," cut into small sections and biologically assayed by the straight-growth method of sectioned Avena coleoptiles, var. Brighton (9). Ultraviolet absorption spectra were obtained after elution of individual spots on the chromatograms.

The presence of 3-indoleacetic acid (IAA) in the acid fraction was indicated by biological assays of spots having R_f values corresponding to synthetic IAA (fig 1 and 2). These spots were further characterized by development of typical colors with Salkowski and Ehrlich spray reagents, and an ultraviolet absorption spectrum characteristic of IAA (major max. at 280 mμ, minor max. at 289 mμ, minima at 245 and 287 mμ, shoulder at 273 mμ). In addition, growth promoting zones, corresponding to two distinct ultraviolet fluorescing spots on the chromatograms at R_f 0.17 to 0.25 and 0.72 to 0.85, were noted (fig. 1). Beside IAA, only one ultraviolet fluorescing spot appeared in chromatograms with water as the solvent (fig 2). The zone corresponding to R_f 0.72 to 0.85 in figure 1 was at the origin in figure 2, and was not biologically assayed.

Growth stimulating zones which fluoresced light blue in ultraviolet were prominent in chromatograms of the "neutral" fractions (fig 3 and 4). The ultraviolet fluorescent spot of R_f 0.70 to 0.90 in figure 3

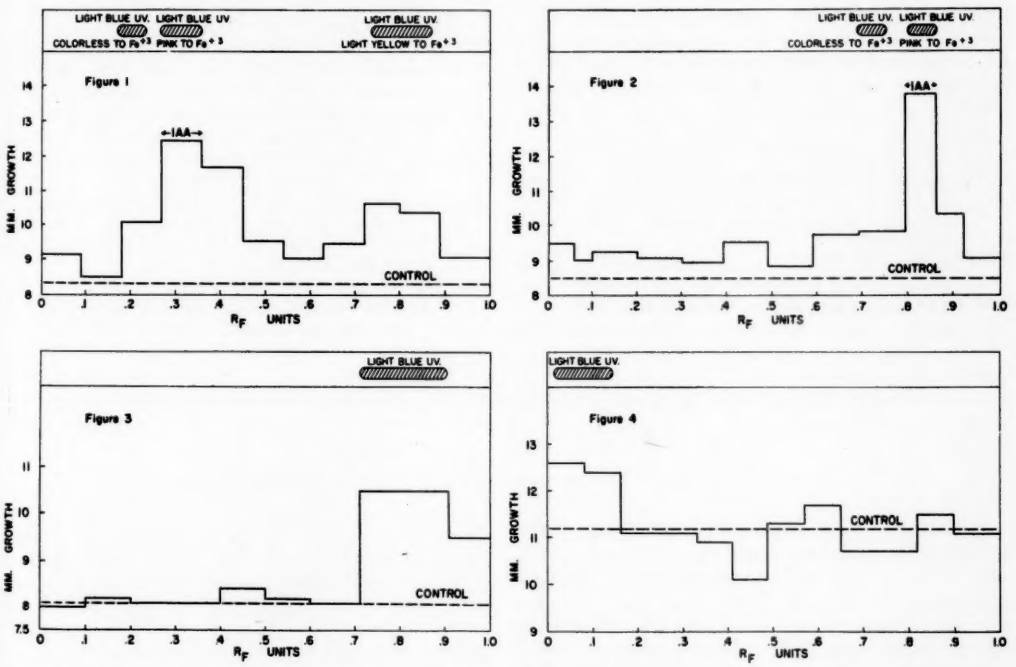

FIG. 1 to 4. Chromatographic separation and bioassay of growth substances in acid and "neutral" fractions of ethyl ether extracts of corn pollen. Upper horizontal bar in each figure represents paper chromatograms after development with solvent and spraying with Salkowski reagent. Vertical bars in histograms (lower part of each figure) represent mean growth of 10 Avena sections on segments of duplicate chromatograms. Each chromatogram represents 8 g of original corn pollen.

FIG. 1. Acid fraction; solvent—2-propanol-ammonia-water (8:1:1 v/v).

FIG. 2. Acid fraction; solvent—water.

FIG. 3. Neutral fraction; solvent—2-propanol-ammonia-water (8:1:1 v/v).

FIG. 4. Neutral fraction; solvent—water.

corresponded to ethyl-3-indoleacetate (R_f 0.81) and 3-indoleacetonitrile (R_f 0.76). However, the zone neither developed typical colors with Salkowski and Ehrlich spray reagents nor possessed an ultraviolet absorption spectrum typical of the indole moiety (see above). The eluate from the growth stimulating zone of R_f 0.00 to 0.16 in figure 4 had an ultraviolet absorption spectrum identical with the eluate from the zone of R_f 0.70 to 0.90 in figure 3 (major max. at 272 $m\mu$, minor max. at 279 $m\mu$, minima at 255 and 277 $m\mu$ shoulder at 265 $m\mu$). This indicates a conjugated system and is very similar to the absorption spectrum of benzoic acid. A "neutral" growth substance with similar properties has been extracted from immature corn kernels in this laboratory (1).

The results of this investigation add evidence to the concept that IAA and other indole derivatives are not the only group of naturally occurring plant growth substances (2, 4).

Summary

In the acid fraction of corn pollen, 3-indoleacetic acid and two other growth promoting substances were observed. The growth regulator in the "neutral" fraction did not possess an indole moiety.

The authors are indebted to Mrs. JoAnne DeVries and Miss Nancy Carson for performing the biological assays.

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UTILIZATION OF D-GLUCURONATE BY CORN COLEOPTILES[1]

W. G. SLATER[2] AND HARRY BEEVERS

Department of Biological Sciences, Purdue University, Lafayette, Indiana

D-Glucuronic acid and D-xylose, which may be regarded as its decarboxylation product, occur naturally in plant cell wall polysaccharides. Although considerable interest has been focused on D-glucuronate metabolism in animals in connection with its role in detoxication little has been established until very recently on its origin and metabolism in plants.

Hough and Pridham (10) have shown that D-glucuronolactone is metabolized in plum mesocarp and this is one of the compounds which Isherwood, Chen and Mapson have shown to function as a precursor for ascorbic acid synthesis in water cress (11). Altermatt and Neish (1) in a paper which appeared after the completion of the present work, have followed the utilization of D-glucuronolactone and several other compounds in the synthesis of cell wall materials in wheat. They have emphasized that D-glucose and D-glucuronolactone are superior to pentoses as precursors of the D-xylose units in xylan, and the labeling data show clearly that loss of carbon 6 is involved in the conversion of D-glucuronolactone to xylan. A similar loss of C-6 from a glucose skeleton was observed in previous work on the origin of the pentose units in wheat xylans (13) and in the pectins of boysenberry and strawberry fruits (16, 17). Some of this work has been reviewed (2).

As a result of their recent work, Altermatt and

[1] Received November 25, 1957.

[2] Present address: Division of Plant Industry, C.S.I.R.O., Canberra, Australia.

Neish propose that uridine diphosphoglucose (UDP-glucose) occupies a central role as the starting point for uridine diphospho-glucuronate and xylan synthesis and for the analogous uridine diphosphogalactose ⟶ araban series of reactions (1).

While investigating the fate of D-glucuronic acid in the plant as part of our studies on carbohydrate catabolism we have found it to be rapidly decarboxylated by some tissues. This paper describes an investigation into features of the decarboxylation and the fate of the 5-carbon residue in the coleoptiles of corn seedlings. It will be seen that the results (which have been described previously in a preliminary form (18) are in accord with the suggestions of Altermatt and Neish (1); indeed we had been led to speculate independently along similar lines.

Methods

In most of the experiments, the coleoptiles of 3-day-old corn seedlings, variety Wf9/38-11 were used. These were obtained by germinating the grain on moist paper at 30° C. The coleoptiles were cut into slices about 0.5 mm thick, and 1 g fresh weight of tissue was incubated with phosphate buffer (pH 5.0 or 7.0) at 25° C in the presence of the appropriate substrate. Large (100-ml) Warburg flasks containing 3 ml of liquid were used, so that the tissue was always well aerated. Oxygen consumption could be measured, and the carbon dioxide was absorbed in CO_2-free sodium hydroxide which was removed for determination of $C^{14}O_2$ and replaced as frequently as necessary during each experiment.

D-Glucuronic acid-6-C^{14} and D-glucuronolactone-6-C^{14} were supplied by the National Bureau of Standards (Dr. H. S. Isbell), and D-glucuronolactone-U-C^{14} (i.e., D-glucuronolactone uniformly labelled with C^{14} in all 6 carbon atoms) was donated by the Corn Products Refining Company. D-Glucuronic acid-U-C^{14} was prepared by hydrolysis of an aqueous solution of the lactone at 100° C for one hour; the acid was then separated from unchanged lactone by paper chromatography in ethyl acetate : acetic acid : water (3 : 3 : 1 v : v : v (5)) and eluted with water.

To determine the radioactivity of the substrates, samples were wet-ashed by the method of Stutz and Burris (20), and the CO_2 absorbed in barium hydroxide. CO_2 released by the tissues was recovered from the alkali by precipitation as barium carbonate, which was then spread as a thin film (4 to 16 mg/sq cm) on planchets, dried, and its activity measured in a windowless gas flow counter. All activity measurements are expressed in counts per minute (cpm) at infinite thinness, i.e., corrected for background and self-absorption.

Results

Ability of Different Tissues to Decarboxylate D-Glucuronic Acid: Table I shows the O_2-consumption and $C^{14}O_2$-production of a number of tissues, sliced thinly and incubated for four hours at 25° C with 50 micromoles (20,600 cpm) of D-glucuronic acid-6-C^{14} at pH 5.0. In general, those tissues with the higher rates of respiration were most efficient in converting the radioactive D-glucuronate to $C^{14}O_2$. The highest activity was shown by young tissues of the three cereals, and the best of these, corn, was used for all subsequent experiments.

Table I

Decarboxylation of D-Glucuronic Acid-6-C^{14} by Various Plant Tissues

	CO_2, cpm *	O_2-uptake **
Corn, coleoptile	2040	1940
Corn, root	1880	1350
Barley, coleoptile	734	930
Wheat, coleoptile,	630	950
Castor bean, hypocotyl	217	1040
Castor bean, endosperm	129	1500
Pea, epicotyl	242	600
Soy bean, epicotyl	224	1000
Lima bean, epicotyl	168	700
Kidney bean, immature seed	314	890
Kidney bean, pericarp	193	320
Tomato, petiole	153	226
Kalanchoe, peduncle	22	295
Bryophyllum, leaf	420	490
Coleus, stem	110	135

* Per g fresh weight in 4 hours.
** Microliters per g fresh weight in 4 hours.
50 micromoles of D-glucuronic acid-6-C^{14} containing 20,600 cpm was supplied as substrate in each case.

Progress of Decarboxylation by Corn Coleoptiles: The course of $C^{14}O_2$ production from 20 micromoles and 50 micromoles of D-glucuronic acid-6-C^{14} is shown in figure 1. With the smaller quantity, half of the radioactivity had been recovered in 16 hours, and 75 % in 21 hours. Respiratory activity declined steadily during the experiment.

Aerobic Nature of the Process: In the absence of air (replaced by nitrogen), production of $C^{14}O_2$ was almost completely inhibited (fig 2).

Origin of CO_2 Produced from D-Glucuronic Acid: In order to determine whether C-6 alone ap-

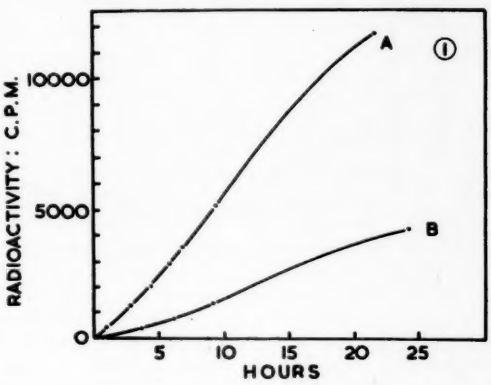

Fig. 1. $C^{14}O_2$ production from D-glucuronic acid-6-C^{14} (A. 50 micromoles; 20,600 cpm: B. 20 micromoles; 5650 cpm) by 1.0 g sliced coleoptiles; pH 7.0.

peared as CO_2, equivalent molar and radiochemical amounts of the specifically (6-C^{14}) and the uniformly labeled acids were used. The specifically labeled acid therefore contained 6 times as much activity in the 6-carbon position as did the uniformly labeled acid, so that if this were the only carbon atom released, a ratio of 6 : 1 in the radioactivity of the CO_2 from the 6-C^{14} and U-C^{14} acids would be expected. This was the value actually observed experimentally, as figure 3 (curves A and B) shows. The values for radioactivity recovered from U-C^{14} (curve A) have all been multiplied by 6 to demonstrate the close agreement of the experimental to the theoretical ratio during the first 12 hours; after that time curve A diverges from B, indicating the release, as CO_2, of carbon atoms other than C-6.

Behaviour of D-Glucuronolactone: D-Glucuronic acid in aqueous solution comes into equilibrium with its corresponding D-glucuronolactone (6). The reaction appears to be a slow one at normal temperatures, although Rabinowitz, in his work on decarboxylation by kidney preparations found that the lactone and acid were equally effective as substrates and considered them to be readily interconvertible (4).

The four curves in figure 3 show the decarboxylation of the free acid and lactone, in both the specifically and uniformly labeled forms, by corn coleoptile tissue. It is clear that the lactone is attacked,

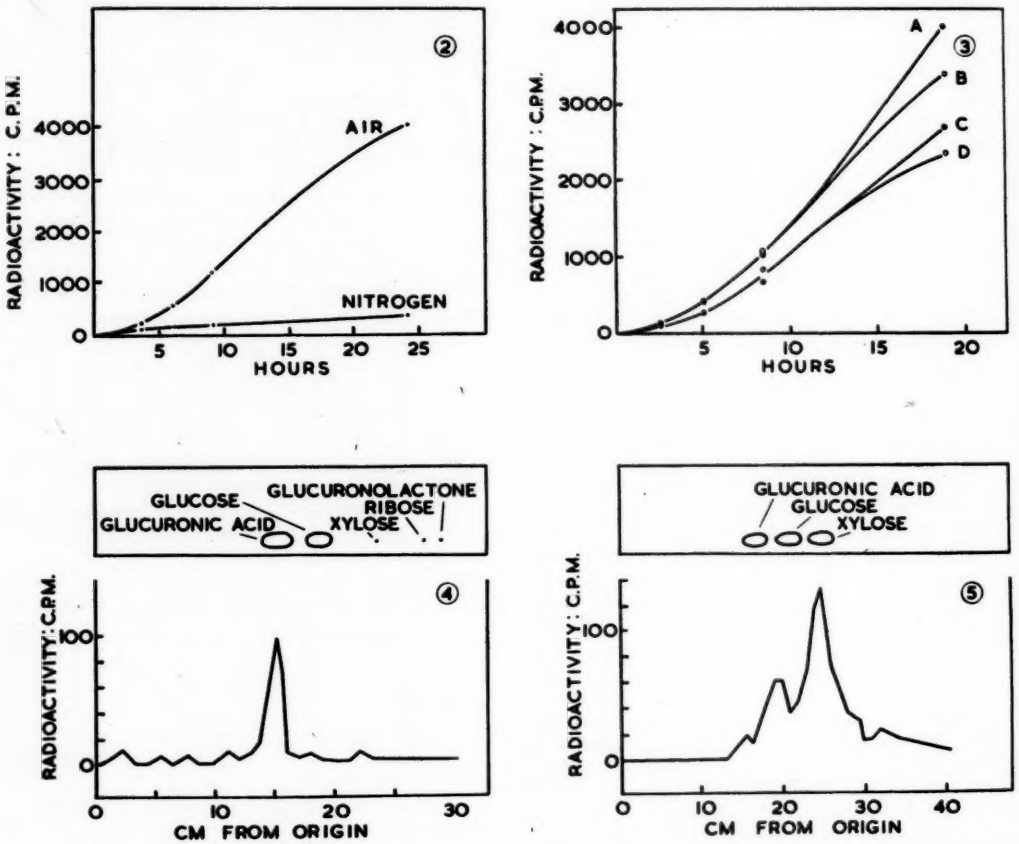

Fig. 2. $C^{14}O_2$ production from D-glucuronic acid-6-C^{14} (20 micromoles; 5650 cpm) in air and in nitrogen by 1.0 g sliced coleoptiles; pH 7.0.

Fig. 3. $C^{14}O_2$ production from 20 micromoles (5650 cpm) of: A. D-glucuronic acid-U-C^{14} (cpm in $CO_2 \times 6$); B. D-glucuronic acid-6-C^{14} (cpm in CO_2); C. D-glucuronolactone-U-C^{14} (cpm in $CO_2 \times 12$); D. D-glucuronolactone-6-C^{14} (cpm in CO_2); by 1.0 g sliced coleoptiles; pH 7.0.

Fig. 4. Chromatogram of alcohol-soluble contents of coleoptiles and medium after incubation with D-glucuronolactone-U-C^{14}. Only D-glucose and D-glucuronic acid were detected in color tests for sugars, as shown in the upper part of the figure.

Fig. 5. Chromatogram of products of hydrolysis from alcoho!-insoluble residue obtained from coleoptile sections after incubation with D-glucuronolactone-U-C^{14}.

although $C^{14}O_2$ release (curves C and D) lags somewhat behind that from the corresponding acids (curves A and B). However, the ratio of radioactivity in the CO_2 from C-6-labeled lactone to that from uniformly labeled lactone was 12 : 1 rather than 6 : 1. The values for $C^{14}O_2$ formation from the D-glucuronolactone-U-C^{14} (C) have been multiplied by 12 to show this relationship. No satisfactory explanation can be offered for the fact that in each experiment in which the two lactones were compared, a ratio of 12 : 1 (rather than 6 : 1 anticipated from the acid results) was obtained. It appears that only half of the added uniformly labeled lactone can be decarboxylated in vivo; in a chemical decarboxylation of the two lactones, achieved by boiling in 1 : 1 hydrochloric acid for 1.5 hours (12a) the radioactivity recovered in the CO_2 from glucuronolactone-6-C^{14} was 6.02 times that from an equivalent amount glucuronolactone-U-C^{14}.

PRODUCTS OF THE DECARBOXYLATION: The product expected after the removal of CO_2 at the 6-carbon atom from D-glucuronic acid is D-xylose. Presumably, this would then be liable to oxidation (3), but the fact that the two curves in figure 3 do not begin to diverge until after some 12 to 15 hours suggests that the residue of five carbon atoms is not, in fact, broken down further during this time. Accordingly, coleoptiles and medium were extracted in boiling 80 % ethanol after an 18 hour incubation with 100 micromoles of D-glucuronolactone-U-C^{14}, and the extract analyzed for soluble products, by paper chromatography in ethyl acetate : acetic acid : water (3 : 3 : 1 v : v : v). A radioactive scanning of the paper was made by cutting it into 1-cm strips and counting these individually without elution; the results of this analysis are shown in figure 4, together with the positions of marker substances on the chromatogram.

In the absence of any trace of pentose, or other derivative of the decarboxylation, other treatments were tried. For example, when the extract was fractionated into acidic, basic, and neutral components on ion-exchange resins, all the activity remained in the acid fraction, and this was found to be entirely due to unchanged D-glucuronic acid itself. Different conditions of extraction, and treatment with phosphatase before extraction (to break down any insoluble phosphorylated derivatives, e.g., of sugars) also proved unfruitful.

Attention was therefore turned to the alcohol-insoluble material. This was strongly radioactive, and a quantitative comparison of the C^{14} in the CO_2 released with that in the residue yielded the results shown in table II in two separate experiments. These figures establish the fact that any 5-carbon product of the decarboxylation is converted rapidly into some insoluble form. An attempt was made to determine which components of the residue were labeled, by hydrolysis and chromatography, but the large excess of inactive cellulose made a quantitative analysis impracticable. Figure 5 illustrates a chromatogram obtained by hydrolyzing the dried alcohol-insoluble residue with 1 % sulphuric acid under reflux for 12 hours (12), and developing the concentrated and neutralized hydrolyzate in ethyl acetate : acetic acid : water. The most strongly radioactive component was D-xylose, with some activity in D-glucose, and a little in uronic acid.

TABLE II

RELATIONSHIP BETWEEN C^{14} IN CO_2 AND IN THE "INSOLUBLE" FRACTION AFTER METABOLISM OF D-GLUCURONOLACTONE-U-C^{14} BY CORN COLEOPTILES

EXTRACTING SOLVENT	CPM IN INSOLUBLE RESIDUE	CPM IN CO_2	RATIO
Hot 80 % ethanol	50,000	9,600	5.2 : 1
Cold 10 % trichloracetic acid	25,200	5,100	4.9 : 1

DISCUSSION

The close adherence to a 6 : 1 ratio in figure 3 (curves A and B) shows that, as in the rat kidney preparations of Rabinowitz (14, 15) it is the carbon atom in position 6 which is lost during decarboxylation of D-glucuronate by corn coleoptile tissue. However, a much greater proportion of the supplied D-glucuronate was decarboxylated in the present experiments than in those of Rabinowitz and Sall (15); whereas the animal preparations effected only 10 to 12 % decarboxylation, the decarboxylation went virtually to completion when 10 micromoles of substrate were supplied to 1 g of corn coleoptiles. Rabinowitz (14, 15) was unable to decide whether the acid or the lactone was the immediate substrate for decarboxylation, because both were equally reactive in his system. In corn, on the other hand, the curve of $C^{14}O_2$ release from the lactone lags slightly behind that for the acid, which might suggest that the lactone is first hydrolyzed. Experiments in which D-glucuronolactone-U-C^{14} was used and the alcohol-soluble products separated chromatographically, showed that corn coleoptile tissue was in fact able to bring about a thermolabile hydrolysis to D-glucuronic acid. At pH 5, where only a trace of acid was produced in the absence of the tissue, hydrolysis was complete within 24 hours when slices were added. At pH 7, where the spontaneous hydrolysis of the lactone occurs at a noticeable rate (about 50 % hydrolysis in 24 hr) no lactone remained after this time in the presence of the tissue, and the radioactive area coincided with D-glucuronic acid (fig 4). No cell-free preparation capable of hydrolyzing the lactone has yet been obtained from corn coleoptiles; Eisenberg and Field have recently described such an enzyme in liver homogenates (7).

The absence of any trace of pentose in the experiments such as that illustrated in figure 4 indicates that the products of decarboxylation are not liberated in the free state, and the high level of radioactivity in the residue supports this suggestion. The products are apparently rapidly converted to insolu-

ble material, of which D-xylose, combined in polysaccharide, is the main radioactive component (fig 5). The presence of D-xylose in a hydrolysis under such drastic conditions as these must be accepted with caution, but there are two reasons for accepting the D-xylose as a valid component of the residue. First, the chromatograms of the digest revealed large amounts of uronic acid which had withstood the acid hydrolysis, but which had very low activity (fig 5). Second, the ratios of (C^{14} in residue) : (C^{14} in CO_2) are so close to theoretical (i.e., 5 : 1) that only traces of radioactive substances, other than the products of decarboxylation, could be present in the insoluble residue (table II).

Existence of an active process such as this in the plant leads to consideration of its significance in vivo. Although free D-glucuronic acid is not known to occur naturally in plants the present experiments show that it is decarboxylated at a rate at least as great as that of D-gluconic acid (3), which as 6-phosphogluconic acid, is an intermediate in the pentose phosphate cycle. It is now suggested that this reaction may throw additional light on the question of pentosan synthesis in plants since it demonstrates convincingly the occurrence in vivo of the split between carbons 5- and 6- of a 6-carbon chain. In addition, it seems clear that the decarboxylation leads quantitatively to xylan synthesis by a process not involving free D-xylose as an intermediate.

These facts are in accord with the following mechanism of xylan synthesis proposed independently by Altermatt and Neish (1) from their extensive work on labeling patterns in the xylan units of wheat polysaccharides.

The facts that the decarboxylation of supplied D-glucuronate has an O_2 requirement and that UDP derivatives of pentose have now been isolated in small amounts from wheat by Ginsburg, Stumpf and Hassid (8) are in harmony with the proposed involvement of UDP derivatives as xylan precursors. The concept derives further support from the work on glucuronyl transfer in animal tissues (19) and from the finding that in the cell-free decarboxylation of D-glucuronate in rat liver, UTP is an effective co-factor (14).

The demonstrated inability of tissues to make direct use of D-xylose in pentosan synthesis (1, 8) must, on this line of reasoning, be ascribed to an inability of the tissue to form directly a UDP derivative. As the labeling data show (1, 8) D-xylose is apparently first converted into hexose units in the pentose phosphate pathway. This hexose unit is then presumably converted to its UDP derivative before oxidation to UDP-glucuronate and decarboxylation to UDP-pentose. This would ascribe the origin of UDP-pentoses to reactions of the corresponding hexose derivatives.

It is implied by the ability of the tissues to utilize D-glucuronate that this can be converted into a UDP form, although no direct evidence for this reaction can be cited. The results from pentose utilization (1, 8) and on uronic acid formation in the cell free system (19, 21) make it appear unlikely that free D-glucuronic acid is an obligatory intermediate in the synthesis of xylans from hexose. The reaction leading to uronic acid products which has been elucidated is one which involves UDP-glucose rather than free glucose, and the enzyme involved is a DPN linked dehydrogenase which yields UDP-glucuronic acid as the product. This reaction has now been demonstrated in pea extracts (21) and the enzyme has been highly purified from this material. The activity of such an enzyme in the present connection could clearly limit the natural rate of xylan (and polyuronide) synthesis from D-glucose. However, it is also clear from the fact that the young tissues had the greater ability to deal with added D-glucuronate that the whole complex of enzymes involved may be more active in the younger, actively growing tissues. In these tissues a significant part of the glucose might be diverted towards pentosan synthesis, and such a diversion leading to an excess of C-6 in the respired CO_2 could well account for occasional values of greater than unity observed for the C-6 : C-1 ratio by Beevers and Gibbs (4).

Summary

Out of a variety of tissues tested, young corn coleoptile was superior in its ability to decarboxylate D-glucuronic acid-C^{14} under aerobic conditions. By comparing radiochemical yields in the $C^{14}O_2$ produced from D-glucuronic acid-6-C^{14} and D-glucuronic acid-U-C^{14} by 1-g coleoptiles, it was shown that, over a period of about 12 hours (during which nearly half of the 20 micromoles of substrate supplied had been utilized) the only carbon from the substrate to appear in the CO_2 was that from C-6. D-Glucuronolactone-C^{14} was also decarboxylated, and the slices were shown to bring about the hydrolysis of the lactone to the free acid.

No soluble products of the decarboxylation of D-glucuronic acid-U-C^{14} could be detected, but the insoluble residue after 80 % alcohol extraction or 10 % TCA precipitation contained five times as much radioactivity as the respired CO_2. Hydrolysis of this material revealed that the bulk of the radioactivity was in D-xylose units derived from polysaccharide. This suggests that xylan synthesis and decarboxylation of D-glucuronate are very closely linked.

Other evidence on the origin of D-glucuronate and its utilization is also summarized, and the present experiments are considered to lend support to some recent proposals of Altermatt and Neish (1) implicating UDP-glucose, UDP-glucuronate and UDP-xylose in xylan synthesis from glucose in plant tissues.

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CHANGES IN OXIDATIVE ENZYME ACTIVITY DURING THE CURING OF CONNECTICUT SHADE TOBACCO[1]

ISRAEL ZELITCH and MILTON ZUCKER

Departments of Biochemistry and of Plant Pathology and Botany,
The Connecticut Agricultural Experiment Station,
New Haven, Connecticut

The activities of enzymes initially present are believed to be responsible for the major chemical changes that occur during the curing of tobacco leaves (3, 10). Accordingly, the stability of specific enzymes under curing conditions could markedly influence the final composition of the cured leaf. Vickery and Meiss (10) and others (3, 4) have shown that the major changes in composition occur during the first 8 to 12 days of air curing. Respiration, which causes a loss of about 16 % of the total organic solids, also ceases by the end of this period (10). However, most previous studies of enzymes have dealt with non-oxidative reactions, and it has been observed that the activities examined persist even beyond 12 days (1, 2, 3, 8). Such results seem surprising since at least 50 % of the proteins are broken down during curing (4, 10). It would appear that proteins of widely different degrees of stability are present and it has recently been suggested (8) that many of the enzymatically active proteins are included in the stable group.

Since oxidations are fundamental to the curing process, the activities of some soluble oxidative enzymes which vary greatly in their mechanism of action have been investigated. A marked decline, and in some cases a complete loss, of enzymic activity as well as a disappearance of oxygen uptake by leaf tissue has been observed. These results correlate well with earlier investigations on the chemical changes that occur during curing.

[1] Received November 30, 1957.

Methods

Sampling and Curing: Leaves were picked by the statistical method (9) from tobacco plants of the shade-grown variety Connecticut 49. Five samples were collected from 10 plants, the 16th to the 20th leaf being taken. All leaves were mature according to commercial standards. Each sample contained 10 leaves. One sample was used to represent the initial time, and the other four were strung on laths and air cured in a chamber maintained at 32° C and 65 % relative humidity with an air velocity of 20 to 25 feet per minute for 2, 6, 9 and 13 days respectively. At the end of each time interval, a sample was withdrawn and weighed, and 1-cm discs to be used for the determination of respiratory activity were punched from identical positions in each leaf. The leaf discs were floated in a small volume of water in Warburg vessels which contained KOH in the center wells, and the rate of oxygen uptake was measured in the dark at 30° C.

Preparation of Extract Containing Soluble Enzymes: After removal of the discs, the leaves were washed with tap water and ground in a chilled plate mill. Sufficient water was added to the cured samples during the grinding procedure to compensate for the water lost during curing. Each extract was filtered through cheesecloth, and collected in the cold in a container to which activated carbon (Darco G-60) had been added to make the final concentration 1 g per 100 g of initial fresh weight of the sample. All subsequent steps were carried out at a temperature between 0 and 5° C. The pH of the homogenate (about 5.5) was raised to about 6.5 by the dropwise addition of 2.5 *N* KOH. The volume, in milliliters, at this stage was approximately the same as the initial fresh weight of the sample in grams. The extract was centrifuged for 10 minutes at 20,000 × G and the supernatant fluid was dialyzed for 20 hours against several changes of distilled water. Turbidity developed during the dialysis, and the insoluble protein was removed by centrifugation for 10 minutes at 20,000 × G. The volume of the supernatant fluid was then measured and samples were used for the enzyme assays. Each assay was carried out at two levels, one twice as high as the other. In order to determine the amount of soluble protein present, a sample of the dialyzed extract was treated with sufficient ethanol in the cold to make the final concentration 75 % by volume. Nitrogen was determined in the precipitate produced.

Malic Dehydrogenase: The assay used was based upon the reaction shown in equation 1 (6).

$$\text{DPNH} + \text{H}^+ + \text{oxalacetate} \rightleftharpoons \text{L-malate} + \text{DPN}^+ \quad (1)$$

To a cuvette of 1 cm light path containing 100 micromoles of tris(hydroxymethyl)aminomethane hydrochloride (TRIS) buffer at pH 7.5, were added 0.3 micromole of reduced diphosphopyridine nucleotide (DPNH), enzyme extract, and water to make the final volume 3.0 ml. The blank cell contained all of the components except DPNH. At zero time, one micromole of oxalacetate was added to each cuvette, and the rate of disappearance of DPNH was measured at 340 mμ in the Beckman model DU spectrophotometer. One unit of enzyme is defined as the amount necessary to cause a decrease in absorbance of 0.01 at 25° C in one minute under the conditions described.

Polyphenol Oxidase: The method was based upon measurement of the rate of formation of dark-colored polymeric compounds by determination of non-specific absorption at 400 mμ (equation 2). To

$$\text{Catechol} + \tfrac{1}{2}\,\text{O}_2 \rightarrow \text{brown pigment} \quad (2)$$

a cuvette containing 100 micromoles of potassium phosphate buffer at pH 6.0, enzyme extract and sufficient water to make the final volume 3.0 ml, were added 20 micromoles of catechol. The rate of "browning" in the interval between 15 and 45 seconds after addition of the substrate was measured in a Beckman model B spectrophotometer. One enzyme unit is defined as the amount required to cause an increase in absorbance of 0.01 in one minute at 25° C. The activity was found to be proportional to enzyme concentration over a 10-fold range.

Diaphorase: The assay was based on measurement in Thunberg tubes of the rate of reduction of 2,6-dichlorophenolindophenol (equation 3) in the

$$\text{DPNH} + \text{H}^+ + \text{dye} \rightarrow \text{reduced dye} + \text{DPN}^+ \quad (3)$$

Coleman model 8 colorimeter at 590 mμ (5). The components of the assay mixture were 100 micromoles of TRIS buffer at pH 7.5, 0.15 micromole of 2,6-dichlorophenolindophenol, enzyme extract, and water to make the final volume 6.0 ml. The side arm of the Thunberg tube contained 0.15 micromole of DPNH. The tubes were evacuated with a water pump, and after a suitable period to permit temperature equilibration, DPNH was tipped in to start the reaction. One unit is the amount of enzyme needed to cause a decrease in absorbance of 0.01 in one minute at 25° C. This is equivalent to about 0.0025 micromole of DPNH oxidized.

Glycolic Acid Oxidase: This activity was determined by the manometric method (11) (equation 4). The Warburg vessels contained 100 micromoles

$$\text{Glycolate} + \text{O}_2 \rightarrow \text{glyoxylate} + \text{H}_2\text{O}_2 \quad (4)$$

of TRIS buffer at pH 8.3, excess riboflavin phosphate, enzyme extract, and water to make the final volume 2.0 ml. Ten micromoles of potassium glycolate were placed in the side arm and KOH was present in the center well. After equilibration at 30° C, the substrate was tipped in and the rate of oxygen uptake was measured for two successive 10-minute periods beginning five minutes after the addition of the glycolate. One unit is defined as the amount of enzyme required to cause an uptake of 1 μl of oxygen in 10 minutes.

Glutamic Dehydrogenase: The oxidation of glutamate was determined by following the rate of formation of DPNH (7) (equation 5). The cuvettes

L-Glutamate + $DPN^+ + H_2O$
$\rightleftharpoons$ DPNH + H^+ + α-ketoglutarate + NH_3 (5)

contained 50 micromoles of TRIS buffer at pH 8.8, 0.9 micromole of disphosphopyridine nucleotide (DPN^+), enzyme extract, and water to make the final volume 3.0 ml. Blank cells contained all components except DPN^+. There was no DPNH formation in the absence of substrate. At zero time, 30 micromoles of potassium L-glutamate were added to each cuvette, and the rate of DPNH formation was measured at 340 mμ in the Beckman model DU spectrophotometer for five minutes. One unit is the amount of enzyme required to cause an increase in absorbance of 0.01 in one minute at 25° C.

Results and Discussion

The average initial fresh weight of the five samples was 262 g (table I) with a coefficient of variation of 0.95 %. The sampling error was thus satisfactorily small. The fresh weight had diminished to less than one half of the initial weight by the 6th day, which coincided with the beginning of the yellow stage. The brown stage had begun by the 9th day, and the leaves of the last sample on the 13th day were completely brown. During the curing, the leaves lost 80 % of their fresh weight; hence the solute concentration was increased about 5-fold in the 13-day period.

As shown in table I, about two thirds of the total soluble protein N disappeared during curing. The figure for the zero time sample is probably too low because of less efficient extraction. The overall decrease agrees well with the losses in total soluble protein that can be calculated from the data of Pogell et al (8). Since the loss of total protein during curing represents about one half of that present initially (4, 10), a disproportionately large change must have occurred in the soluble protein fraction which is known to include many oxidative enzymes.

The oxygen uptake by the leaf discs diminished slowly for the first two days, but decreased rapidly thereafter, and was barely detectable by the 9th day. At the end of the yellow stage, when browning begins, it seems safe to assume that death of the plant cells had occurred. This time also corresponds to the point after which little further loss of total organic solids occurs (10).

Table I

Changes in Composition and Respiration Rate During Curing

Curing time	Fresh weight	Total soluble protein N	Oxygen uptake
days	*g*	*mg*	*μl/15 discs × hr*
0	262	101	60.1
2	198	121	52.3
6	125	101	20.6
9	78	40.9	2.8
13	50	36.8	0.0

Table II

Oxidative Enzyme Activity of Initial Sample

Enzyme activity	Volume of extract assayed	Arbitrary units
	ml	*per ml* *
Malic dehydrogenase	0.002	2,800
	0.004	2,930
Polyphenol oxidase	0.10	76.0
	0.20	84.5
Diaphorase	0.005	1,140
	0.01	1,090
Glycolic acid oxidase	0.10	221
	0.20	194
Glutamic dehydrogenase	0.05	34.4
	0.10	35.4

* To convert arbitrary units per ml to total units per sample, multiply by 290, the final volume of the extract in ml.

The sensitivity of the enzyme assays and the activities of the initial sample are shown in table II. The reproducibility of the assays at two levels of enzyme concentration supports the validity of the activity measurements.

The changes in oxidative enzyme activity are plotted in figure 1, the assay at zero time being taken as 100 %. Malic dehydrogenase and polyphenol oxidase are quite stable for the first two days, but activity is lost rapidly thereafter so that by the 13th day only 23 and 13 %, respectively, remain. Diaphorase decreases in a more regular fashion, and only 13 % of the initial activity was found at the 13th day of curing. Glycolic acid oxidase and glutamic dehydrogenase are much less stable than the others, 1 and 4 % of the initial activity, respectively, remaining on the 9th day, and no activity being detectable by the 13th day with the sensitive assays used.

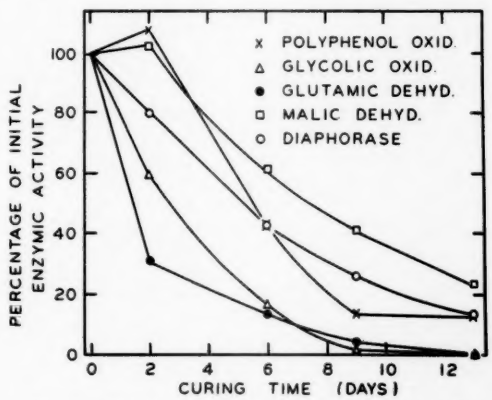

Fig. 1. Relative changes in activity of polyphenol oxidase, glycolic acid oxidase, glutamic dehydrogenase, malic dehydrogenase, and diaphorase during curing.

TABLE III

SPECIFIC ACTIVITY OF OXIDATIVE ENZYMES DURING CURING *

CURING TIME IN DAYS	MALIC DEHYDROGENASE	POLYPHENOL OXIDASE	DIAPHORASE	GLYCOLIC ACID OXIDASE	GLUTAMIC DEHYDROGENASE
0	8,220	230	3,200	596	100
2	7,090	207	2,140	294	21.2
6	5,020	97.8	1,370	92.7	13.8
9	8,360	74.8	2,050	21.6	10.6
13	5,220	79.9	1,150	0.0	0.0

* Expressed as arbitrary units per mg of protein N.

Since much of the soluble protein disappears during curing (table I), it is of interest to compare the various enzymic activities in terms of protein N. It is clear from table III, that malic dehydrogenase activity decreased at about the same rate as the soluble protein during curing, since the specific activity did not change appreciably. Diaphorase, polyphenol oxidase, glutamic dehydrogenase, and glycolic acid oxidase activities, however, decreased more rapidly than the soluble protein, a result which emphasizes the unstable nature of these oxidative enzymes.

The technique used for the extraction of soluble protein is necessarily arbitrary, and the possibility existed that the losses in enzymic activity observed were caused by an unsatisfactory extraction procedure or by a change in the solubility of the enzyme rather than by an alteration in the enzyme protein. Glycolic acid oxidase can be conveniently assayed in the crude homogenate, and a comparison was therefore made of the activity recovered in the final enzyme extract with that originally present (table IV). There was essentially no difference in the recoveries compared with the 40-fold decrease in activity of glycolic acid oxidase between the 2nd and the 9th day of curing; hence it can be concluded that the loss in activity of glycolic acid oxidase was not attributable to an inefficient extraction procedure or to a change in its solubility. An inactivation of the enzyme must have occurred.

At least some oxidative enzymes do not survive air curing, and a considerable variation in stability is exhibited by different enzymes. The overall similarity of the loss in oxidative enzyme activity to the loss in the rate of oxygen uptake (table I) suggests that respiration ceases during curing because inactivation of oxidative enzymes accompanies the breakdown of protein and dehydration of the tissues. The loss both in respiration and enzymic activities shown confirms results obtained from a similar experiment carried out one year earlier.

TABLE IV

RECOVERY OF GLYCOLIC ACID OXIDASE ACTIVITY IN ENZYME EXTRACT

CURING TIME	TOTAL GLYCOLIC ACID OXIDASE ACTIVITY		RECOVERY
	HOMOGENATE	ENZYME EXTRACT	
days	*arbitrary units*		*%*
2	63,600	35,600	56
6	11,700	9,360	80
9	1,170	883	75

It seems clear that many of the soluble proteins involved in respiration are altered during curing. The present studies show that some of the critical enzymes remain functional for limited periods of time only and lead to the conclusion that the rate of enzyme inactivation controls the final composition of the cured leaf. This is an inference which may be helpful in developing improved conditions for the process.

SUMMARY

The rate of oxygen uptake by intact tobacco leaf tissue and the activities of malic dehydrogenase, polyphenol oxidase, L-glutamic acid dehydrogenase, diaphorase, and glycolic acid oxidase in extracts have been studied at intervals during the process of air curing. Both the enzymic activities and the respiration rate decreased rapidly during the first 13 days, and it is suggested that the major oxidative changes involved come to a halt as a result of inactivation of the enzymes. Many of the soluble proteins including certain enzymes undergo alteration during curing. These observations throw light upon some of the results described in previous chemical investigations of the curing process.

Grateful acknowledgment is made to Mrs. Marcia W. Sutter and Miss Edna Baker for skillful technical assistance, to Dr. H. B. Vickery for helpful discussion, and to the National Science Foundation for a grant which supported a part of the expense of this investigation.

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OBSERVATIONS ON THE INCORPORATION OF C^{14} INTO TARTARIC ACID AND THE LABELING PATTERN OF D-GLUCOSE FROM AN EXCISED GRAPE LEAF ADMINISTERED L-ASCORBIC ACID-6-C^{14} [1,2]

FRANK A. LOEWUS and HELEN A. STAFFORD

Western Regional Research Laboratory,[3] Albany, California, and
The Biology Department, Reed College, Portland, Oregon

Vickery and Palmer (12) have speculated on the possible precursor relationship of D-glucose to (+)-tartaric acid in plants. C-2 and C-3 of D-glucose have the same configuration as C-2 and C-3 of (+)-tartaric acid. They proposed a metabolic path in which C-5 and C-6 were lost, presumably via an intermediate such as 5-keto-gluconic acid.

Hough and Jones (9) have stated that hexuronic acids, L-ascorbic acid, and (+)-tartaric acid commonly occur together in nature. Conceivably, L-ascorbic acid might be the precursor of (+)-tartaric acid through loss of C-1 and C-2 of the former and subsequent oxidations of the terminal carbons of the four carbon fragment to carboxyl groups. C-4 and C-5 of L-ascorbic acid have the same configuration as (+)-tartaric acid.

We have attempted to test the latter possibility. L-Ascorbic acid-6-C^{14} [4] (3 mg containing 4.7 μc of C^{14}) was fed through the cut stem to a single grape leaf (Mission variety, second leaf from the tip of an actively growing vine) in 0.3 ml of distilled water. The leaf was illuminated by a pair of 10-watt daylight fluorescent bulbs at a distance of 12 cm. Practically all of the radioactive solution was taken up in five hours. After 8 hours, the leaf was placed in a closed 350-ml container in the dark for an additional 17 hours. Finally, the accumulated respiratory CO_2 was aspirated into a gas trap of N NaOH. Approximately 5 % of the administered label was lost as CO_2 during the dark period.

The soluble constituents of the leaf were separated and recovered as described in another paper (11). Most of the activity remained in the particle-free extract. Very little activity was removed during passage through a cationic exchange resin (Dowex 50, H^+). About one half of the activity remained on the anionic exchange column (Dowex 1, formate). Most of this activity was eluted with a 3 N formic acid gradient (10) in four peaks. The 1st peak coincided with the first traces of acid through the column and might be due to inadequate washing of the column after loading. The 2nd, a very narrow sharp peak, came in the region characteristic of ascorbic acid. Two peaks of lesser activity followed, the last corresponding to malic acid on a paper chromatogram (10). The tartaric acid peak, located by its acid titration curve and by its ammonium metavanadate reaction, had such low activity as to be undetectable by the solid sample counting procedure employed. After three recrystallizations, the potassium acid tartrate from this peak had a specific activity of 24 cpm per mg of carbon (gas-phase counter, 80 % efficient). This amount of C^{14} was too low to be of any significance as concerns the possible metabolic conversion of ascorbic acid to tartaric acid in the grape leaf. The fact that some activity did enter the tartrate molecule from C-6 of L-ascorbic acid suggested that this label was derived, indirectly perhaps, from the sugar pool of the leaf.

In order to explore the latter possibility, the neutral effluent of the Dowex 1 column was concentrated in vacuo to a thick sirup and chromatographed on paper using a solvent composed of ethyl acetate, pyridine, and water (8 : 2 : 1) (13). About 50 % of the C^{14} remained very close to the origin after development. Another 30 % was found in the sucrose. About 5 % each was found in the glucose, fructose, and xylose bands. The glucose region, which was free of other reducing sugars and showed a single darkened area in the corresponding radioautogram, was cut out and eluted. About 0.7 mg of glucose was recovered. It had a specific activity of 100,000 cpm per mg of carbon as determined by wet combustion of an aliquot after dilution with unlabeled D-glucose.

[1] Received December 9, 1957.
[2] Work done at the Western Regional Research Laboratory, Albany, California.
[3] Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.
[4] Kindly supplied by Dr. H. S. Isbell, National Bureau of Standards, Washington, D. C.

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN THE D-GLUCOSE RECOVERED FROM A GRAPE LEAF LABELED WITH L-ASCORBIC ACID-6-C^{14}

CARBON ATOM OF GLUCOSE	PERCENT OF TOTAL ACTIVITY
1	21.5
2	6.0
3	23.0
4	21.0
5	2.5
6	26.0

This activity was about one tenth of the specific activity of the original L-ascorbic acid-6-C^{14}. The distribution of this activity as determined by degradation with *Leuconostoc mesenteroides* (7) is given in table I. Carbons 1, 3, 4, and 6 were almost equally labeled and together accounted for over 90 % of the total activity of the glucose. A significantly greater amount of label appeared in C-2 than in C-5.

The results indicate that L-ascorbic acid-6-C^{14} was extensively metabolized in the grape leaf and that C-6 readily entered the triose phosphate pool in such a manner that label in the terminal carbons of the triose became equilibrated. It has recently been reported that in the guinea pig (3) ascorbic acid is metabolized by oxidation to diketogulonic acid and subsequent decarboxylation. The L-xylose formed in this reaction could enter into the pentose metabolism of the animal via a conversion to D-xylulose (8) which could then be metabolized by the pentose phosphate pathway (2) to hexose. If such processes of conversion exist in the plant, then it would be quite easy to visualize a pathway leading to the observed labeling pattern. Label from L-ascorbic acid-6-C^{14} would first be converted to L-pentose-5-C^{14}, hence to D-pentose-1-C^{14} (an inversion of the carbon chain would occur with the intermediate polyol formation), and then by pentose phosphate metabolism to hexose phosphate-1,3-C^{14} (1, 6). The latter, upon equilibration with the triose phosphate metabolism of the leaf would give rise to the observed D-glucose labeled primarily in carbons 1, 3, 4, and 6 (5). The slightly greater amount of label in C-2 than in C-5 would be a consequence of pentose phosphate metabolism (1, 4).

The facile incorporation of label from L-ascorbic acid-6-C^{14} into the carbohydrate metabolism of the grape leaf probably accounts for the small amount of label observed in (+)-tartaric acid (11). There does not appear to be a direct pathway for the formation of (+)-tartaric acid from L-ascorbic acid in the grape leaf.

The authors are indebted to Miss Rosie Jang for her assistance in the glucose degradation.

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Dr. Sterling B. Hendricks, distinguished plant physiologist, member of the Editorial Board of this Journal, trustee and past vice-president of our Society, has received the highest honor the U.S. can bestow upon career civilian employees. On 16 January the White House announced Dr. Hendricks as one of five recipients of the President's Award for Distinguished Civilian Service. Recipients of the award, established by the Congress in 1956, are recommended by an awards board headed by Secretary of Labor J. P. Mitchell. A gold medal was presented to Dr. Hendricks at a White House ceremony on 27 January in recognition of "his discoveries in soil clays, phosphate minerals, radioisotopes, plant physiology, and fundamental chemistry." Dr. Hendricks is "one of the most distinguished and honored scientists of our time," the White House said. Congratulations, Sterling Hendricks.

INTRODUCTION TO ENZYMOLOGY—Alan H. Mehler. Academic Press, Inc., New York. 423 pages. 1957. $10.80.

This book has evolved from a course of lectures given by Dr. Mehler of the National Institutes of Health to a group of investigators with different biological and chemical backgrounds. It will therefore evoke interest as a possible text or reference work to be considered alongside other books in the field of enzymology, which, it will be admitted, are still few in number and not all equally up-to-date or comprehensive. The present book is admirably up-to-date and it provides a considerable amount of detail about a great number of individual reactions, but these of course are not the only considerations on which a book written as an *introduction* to the subject should be evaluated.

The author's approach has not been to treat individual classes of enzymes separately, but to deal with them seriatim as they occur in various well recognized metabolic sequences. The development of general concepts about enzymes which was the result being aimed for, has not in the outcome been fully achieved. To be sure, at appropriate points in the text, the properties of this or that enzyme are treated at length to emphasize some particular aspect of enzymology, but the impression which was gained at first reading was one of numerous very well documented metabolic reactions rather than one of the ingenious catalysts themselves which can bring about these reactions at appropriate rates. The procedure adopted of writing the reactions with structural formulae of the reactants but without the name of the enzyme responsible for catalyzing the reaction, heightens this impression.

After a very brief introduction there follow sections on the hydrolysis of proteins, fermentation and oxidation, biological oxidation, sugars, polynucleotides, amino acids, and acids and acid derivatives. A short chapter on the organization of structure and function, which includes a section on the synthesis of proteins, completes the volume. Sections on oxidation-reduction potentials and on free energy and the concept of bond energy are included, but surprisingly, the former comes after the treatment of glycolysis and pyruvate oxidation and the latter between "pyridine nucleotides" and "flavoproteins." The type of treatment which was adopted has not allowed the inclusion of chapters which an introduction to the subject might logically be expected to have. For example there is no general treatment of methods of preparation, separation and purification, of assay procedures or of the use of enzymes as analytical tools or in technology.

The text is written in an economical and terse style and a justifiably cautious note is struck throughout. For example the author is careful to emphasize that individual enzymes prepared from different sources may have rather different properties; he is chary of enzyme models, he gives examples of the shortcomings of crystallinity as the final criterion for purity, and a skeptical view is taken of what some may consider progress in the field of protein synthesis. A few minor errors and inconsistencies were noticed but this is not surprising in a book with so much detail.

The reader who is a plant physiologist will perhaps be reconciled to his finding that in the overall biochemical picture provided by the book, work with enzymes from higher plants occupies, rightfully, a rather small part. Nevertheless it may appear surprising that, for example, glycolic oxidase and glyoxylic reductase, which have been obtained in highly purified form are hardly mentioned and that the phosphorylating abilities of illuminated chloroplasts are not given greater prominence. Two interesting activities of enzymes (not from plants) which did not find a place in the book are those concerned with luminescence and with light reception in the eye.

It might well be argued that what have been construed as shortcomings in the foregoing derive directly from the manner of treatment adopted and that it is good discipline, even for the beginner, to be made to assimilate important facts about enzymes and arrive at general concepts about enzyme action while the reactions of metabolic sequences are being discussed in detail. However, it seems likely that among the many who will find the book of value, the most active users will not be the uninitiated, for whom the book was intended, but those who, by virtue of some experience in the field are already predisposed to including "information about enzymes in their scientific thinking".—H. BEEVERS, *Dept. of Biological Sciences, Purdue University, Lafayette, Indiana.*

DIE BIOLOGIE DER BLUTE—F. Knoll. 164 pages, 79 illustrations. Volume 57 in Verstandliche Wissenschaft; DIE SAFTSTROME DER PFLANZEN—B. Huber. 126 pages, 75 illustrations. Volume 58 in Verstandliche Wissenschaft; DIE ENTSTEHUNG DER KULTURPFLANZEN—F. Schwanitz. 151 pages, 59 illustrations. Volume 63 in Verstandliche Wissenschaft. Published by Springer-Verlag, Berlin. Price of each volume DM 7.80.

The German pocket series "Verstandliche Wissenschaft" contains a number of titles in the botanical field. The three books reviewed here are indicative of the high level of excellence maintained in this series. The authors are well known and they have written thoughtful, intelligent accounts of the status of knowledge in their own fields of interest. The books are intended for the well-informed layman but scientific accuracy is not sacrificed. These are very readable books.

"Die Biologie der Blute" describes the structure and function of the flower. The structural modifications of flowers as well as the various pollinating agents are presented in a most interesting manner. The text is accompanied by numerous illustrations.

In "Die Saftstrome der Pflanzen" Dr. B. Huber very ably summarizes the present state of information regarding the movement of water and solutes in plants. He first describes the anatomical features of the plant and then discusses the experimental evidence leading to the various theories of sap movement.

"Die Entstehung der Kulturpflanzen," by Dr. F. Schwanitz, is a very interesting account of the factors concerned in the development of cultivated plants. The transition from wild plants to cultivated plants is first discussed and then the many ways of modifying and improving our present-day cultivated plants are discussed.—G. R. NOGGLE, *University Station, Gainesville, Florida.*